THE CONVERSION OF D- AND L-TRYPTOPHAN TO INDOLE COMPOUNDS IN THE RAT

By

NORMAN CHEA-SAN CHEN

Bachelor of Science
Taiwan Provincial Chung-Hsing University
Taichung, Taiwan
1958

Master of Agriculture
The University of Tokyo
Tokyo, Japan
1964

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
August, 1969

THE CONVERSION OF D- AND L-TRYPTOPHAN

TO INDOLE COMPOUNDS IN THE RAT

Thesis Approved:

Robert K. Gholson
Thesis Adviser

Roger E. Koeppe

Kurt E. Ebner

Elizabeth T. Handy

George R. Waller

D. D. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author gratefully acknowledges the very competent guidance, endless patience, continual advice and encouragement of Dr. Robert K. Gholson as major professor.

Special thanks are due to Dr. R. E. Koeppe, Dr. G. R. Waller, Dr. K. E. Ebner, and Dr. E. T. Gaudy for their contributions and their valuable time spent as members of the advisory committee.

Sincere appreciation is extended to Dr. J. L. Chandler for his valuable discussions. Also, thanks are due to fellow members of the research group of Professor R. K. Gholson for their help.

A very special appreciation is extended to his wife, Fuh-mei, for her encouragement, understanding, and patience exercised during the years of graduate study. The encouragement from his parents and parents-in-law on his studies in the United States is also appreciated.

Finally, the author is indebted to the Department of Biochemistry for providing research facilities and especially to the American people who, through their agencies, the National Institutes of Health, and the National Science Foundation, provided the financial support which made this study possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

B_6GFD: Urinary Radioactive Metabolite of B_6-Deficient Germ-Free Rat Injected with D-Tryptophan-Methylene-^{14}C.

B_6GFL: Urinary Radioactive Metabolite of B_6-Deficient Germ-Free Rat Injected with L-Tryptophan-Methylene-^{14}C.

B_6LN: Urinary Radioactive Metabolite of B_6-Deficient Rat Injected with L-Tryptophan-Methylene-^{14}C.

GFD: Urinary Radioactive Metabolite of Germ-Free Rat Injected with D-Tryptophan-Methylene-^{14}C.

GFL: Urinary Radioactive Metabolite of Germ-Free Rat Injected with L-Tryptophan-Methylene-^{14}C.

IAA: Indoleacetic Acid

I-ald: Indole-3-Carboxaldehyde

I-CN: Indole-3-Acetonitrile

I-gly: Indoleaceturic Acid

K. A.: Kynurenic Acid

Kyn: Kynurenine

LN: Urinary Radioactive Metabolite of Normal Rat Injected with L-Tryptophan-Methylene-^{14}C.

N-A-Kyn: N-Acetylkynurenine

N-A-OH-Kyn: N-Acetyl-3-Hydroxykynurenine

OH-IAA: 5-Hydroxyindoleacetic Acid

OH-Kyn: 3-Hydroxykynurenine

OH-T-NH_2: Serotonin

OH-Try: 5-Hydroxytryptophan

TEA-F: Triethylamine-Formate Buffer Solution

$T\text{-}NH_2$: Tryptamine

Try: Tryptophan

X. A.: Xanthurenic Acid

CHAPTER I

INTRODUCTION

In 1908, Herter (1) first isolated the chromogen of the urorosein reaction (a red color with hydrochloric acid and nitrite) from urine and identified it as indoleacetic acid (IAA). The original identification of IAA as a growth factor for plants was made by Kogl et al. (2) on material isolated from human urine. Its formation in plants established it as a substance of great metabolic consequence. The first indication of its origin was reported by Thimann (3), who found that tryptophan was needed in the medium of Rhizopus for auxin production. The existence in plants of enzymes (4, 5, 6) which form IAA from tryptophan has also been reported. In spite of the fact that the potent effect of this material on plant growth has been known for more than 35 years, it is only since the rapid development of chromatographic and radiochemical techniques during the last fifteen years that increasing attention has been paid to the biochemistry of indole compounds in plants, animals, and bacteria. Owing to the development of these techniques, a number of experiments have amply confirmed the view that tryptophan is the source of indoleacetic acid (IAA) in plants (7, 8), in animals (9, 10), and in bacteria (11, 12).

In man, IAA and indoleaceturic acid have been observed as excretory products in urine for many years. It has been generally assumed that these compounds were produced from tryptophan by the animals'

microbial flora (13). The IAA is derived from bacterial degradation of tryptophan in the gut and is then absorbed into the blood stream and to a large extent conjugated with glycine in the liver. Thus excretion can be increased in the presence of an abnormal intestinal flora (13). However, it was found that intraperitoneal injection of DL-tryptophan-7a-^{14}C resulted in excretion of IAA-^{14}C (9) and an enzyme system capable of converting L-tryptophan to IAA was observed in mammalian tissues (10). These findings suggest that formation of IAA from tryptophan may be a normal metabolic pathway in mammals.

It has also been reported that conversion of DL-tryptophan-7a-^{14}C to IAA and indoleaceturic acid is greatly increased in vitamin B_6-deficiency in rats (9). A possible explanation for this observation is that IAA is derived chiefly from D-tryptophan and that vitamin B_6-deficiency interferes with the conversion of D-tryptophan to L-tryptophan.

This dissertation is concerned with studies designed to elucidate the mode of action of vitamin B_6 on the production of IAA. The quantitative and qualitative evaluation of the excreted indole compounds which are derived from D-tryptophan or L-tryptophan provide some indirect evidence concerning the pathways involved in the metabolic interconversion of indole compounds in mammals. The isolation of any new metabolites of tryptophan from the urine of rats injected with tryptophan, using improved separation techniques, was also the purpose of this research.

Since a satisfactory separation system was not available, this research was at first devoted to developing an efficient column chromatographic procedure for the separation of urinary indole and other

tryptophan metabolites. A dual DEAE-cellulose column chromatography system using triethylamine-formate (TEA-F) buffer has been developed and used in this research.

CHAPTER II

LITERATURE REVIEW

The Metabolism of D-tryptophan

L-tryptophan can be converted to the DL-form by heating with baryt-water at 150°C for 5-6 hours, or by acetylation at room temperature. In 1933, Berg (14) isolated D-tryptophan from the quinine-salt of the DL-form in methanolic solution. Woods (15) reported that half as much indole was formed from DL- as from L-tryptophan by Escherichia coli. Subsequently, Majima (16) obtained D-tryptophan from the DL-form using E. coli. More recently D-tryptophan conjugated as N-malonyl-D-tryptophan has also been isolated from a large number of fruits and vegetables (17). The same nutritional effect of the D-isomer as the L-form in animals has been known for many years (18, 19). However, D-tryptophan has lower nutritional values than the corresponding L-isomer (19, 20). D-tryptophan is not converted to indole by E. coli, but can replace L-tryptophan in nutritional tests in animals. This suggests that the D- and L-isomers can be interconverted in animals. In 1937, Kotake and Goto (21) found that E. coli can form indole from some substance in the deproteinized supernatant of a reaction mixture containing D-tryptophan and liver homogenate. They suggested that D-tryptophan is converted to the L-form in the liver. Also Behrman and Cullen (22) and Martin and Durham (23) have shown that D-tryptophan is converted by their bacterial preparations to L-tryptophan prior to

further metabolism. On the other hand, a metabolic pathway for D-tryptophan that involves D-kynurenine has been suggested by studies in bacteria (24, 25), and in mammals (26, 27). However, Loh (28) has demonstrated that D-tryptophan can be either converted to D-kynurenine by enzymes specific for the D-isomer or be converted to 3-indolepyruvic acid and L-tryptophan and then to L-kynurenine in mammalian tissues. The enzyme which converts D-tryptophan to D-kynurenine was located in the soluble fraction of liver homogenate (29).

Recently, Higuchi and Hayaishi (29) have summarized the significant differences between L-tryptophan pyrrolases and rabbit intestinal D-isomer pyrrolase in their behavior toward the D- and L-enantiomorphs of tryptophan. D-tryptophan pyrrolase activity was markedly inhibited by L-tryptophan, whereas L-tryptophan pyrrolase was not inhibited by D-tryptophan. D-isomer pyrrolase was capable of oxidation of L-tryptophan at a limited rate, but the L-isomer pyrrolases of liver and bacteria were completely inactive with D-tryptophan as a substrate. Moreover, the conversion of D-tryptophan to D-kynurenine required a formamidase reaction to hydrolyze the presumed intermediate compound, D-formylkynurenine, to yield D-kynurenine and formic acid.

Biosynthesis and Metabolism of Indoleacetic Acid, Indole-3-carboxaldehyde and Related Compounds

Indoleacetic acid (IAA) is well known as a metabolic product of tryptophan in plants, in microorganisms, and in animals. Yet the pathways of IAA biosynthesis and its metabolism in higher and lower plants are still incompletely understood. In animals, the pathway for IAA synthesis is even more obscure than in plants.

Since IAA is well established as a principal hormone of higher plants, the study of the biochemistry of IAA and related compounds has been carried out extensively in the higher plants. In 1944, Larsen (30) reported the isolation and partial purification of a neutral indole compound with auxin activity from the tissues of pea, sunflower, and broadbean plants. From its chemical properties, he concluded that the substance was 3-indoleacetaldehyde. Evidence for the occurrence of this neutral compound in certain other plants was later provided by Hemberg (31) and by Gordon and Nieva (32, 33). More convincing proof of the presence of this substance in pea plants has been provided by Larsen and Aasheim (34). In 1953, another neutral indole compound, 3-indoleacetonitrile, was found in cabbage plants by Henbest et al. (35). Larsen (36) demonstrated that exogenously supplied 3-indoleacetaldehyde was converted to IAA by oat coleoptile tissue. Also Thimann (37) demonstrated the conversion of a synthetic sample of 3-indoleacetonitrile to IAA in several plant tissues. Recently, Moor and Shaner (38, 39) provided evidence that IAA is synthesized from tryptophan-^{14}C in cell free extracts of shoot tips of green pea seedling via indolepyruvate and indoleacetaldehyde. They demonstrated that the enzymic conversion of tryptophan-^{14}C to IAA was enhanced by the addition of pyridoxal phosphate, α-ketoglutaric acid and thiamine pyrophosphate to reaction mixtures. The postulated intermediates, indolepyruvic acid-^{14}C and indoleacetaldehyde-^{14}C were also isolated from the cell-free reaction mixtures (38, 39).

During studies of indoleacetic acid in plants, it has been learned that plants infected by the vascular pathogen Pseudomonas solanacearum E. F. Smith manifest symptoms associated with increased auxin content.

The role of IAA in the development of these symptoms has been the subject of intensive study. In tobacco, it has been shown that the concentration of IAA in diseased tissues may be 100 times that in healthy tissues (40). It has been suggested that growth responses and changes in respiratory metabolism are due to hyperauxiny (40).

Due to the connection between pathogens and host plants, IAA has also been studied intensively in microorganisms. So far, the presence of a tryptophan transaminase in bacteria has been reported for E. coli (41), Pseudomonas (44, 45) and Clostridium sporogenes (46). Thus, the so-called indolepyruvate pathway receives frequent mention in bacterial systems. A large number of microorganisms appear to first deaminate tryptophan to indolepyruvic acid, which is then decarboxylated to indoleacetaldehyde (47, 48, 49, 50). In others, synthesis of IAA may proceed via 3-indoleacetamide (44, 51) or via decarboxylation of tryptophan to tryptamine (52).

In contrast with the flourishing studies of IAA in plants and in microorganisms, there have been few studies of IAA metabolism in animals. Although Werle and Mennicken (53) reported over thirty years ago that mammalian tissues can decarboxylate L-tryptophan, until recently few investigators regarded tryptamine as a normally occurring substance in tissues. Its presence in normal urine reported by Rodnight (54) in 1956, followed by the demonstration of tryptophan decarboxylation in animal tissues in 1959 by Weissbach et al. (10), has clearly established that tryptamine must be a normal constituent of animal tissues, and may be one of the intermediates in the formation of IAA from tryptophan. In animals, two metabolic pathways of tryptophan leading to IAA have been proposed (10); one via indolepyruvic acid, the

other via tryptamine. However, the bulk of IAA probably arises from transamination, with only a smaller portion arising through decarboxylation (10).

IAA has been frequently found as conjugated compounds; as indoleacetyl aspartate in plants (55) and as indoleaceturic acid (glycine conjugate) in urine (13). Zenk (56) suggested that indoleacetyl-CoA was the intermediate in the enzymatic formation of indoleacetyl-amino acid conjugate compounds. More recently, indoleacetyl-ε-L-lysine was isolated from Pseudomonas savastanoi (57).

It has been known that IAA may be further metabolized to other indole compounds. Some of the indole compounds found in plant materials undoubtedly come from IAA. Indole-3-carboxaldehyde has been reported as a product of the action of ultraviolet light on IAA (58, 59, 60). Another photochemical product of IAA was also isolated and identified as indole-3-glycolic acid (58). Fischer (58) accordingly formulated the photo-oxidation of IAA as

$$\text{Indole-CH}_2\text{-COOH} \longrightarrow \text{Indole-CHOH-COOH} \longrightarrow \text{Indole-CO-COOH} \longrightarrow \text{Indole-CHO}$$

An enzyme responsible for the oxidation of IAA has been found in a number of higher plants (61, 62, 63, 64, 65) and fungi (66). Indole-3-carboxaldehyde has been identified as the metabolic product of this enzymatic reaction (61, 62, 63, 64, 65). Indole-3-carboxaldehyde has also been isolated in significant amounts from human adrenal tumor tissue (67). More recently, Glombitza (68) has isolated this compound from yeast incubated with tryptophan, and Iskrić and Kveder (69)

reported this compound was formed from tryptamine in a rat liver preparation.

Finally, several pathways have been suggested for the conversion of tryptophan to IAA and the related indole compounds in plants (70), in microorganisms (51) and in animals (10). These pathways are shown in Figures 1, 2, and 3, respectively.

Two Other Pathways Leading to Indole Compounds From Tryptophan

Fission to Indole

There are at least four major metabolic pathways of tryptophan: (a) oxidation to formylkynurenine; (b) hydroxylation to 5-hydroxy-tryptophan; (c) conversion to indoleacetic acid; (d) fission to indole, pyruvate and ammonia. Of these four pathways, three form indole compounds from tryptophan.

A quarter of a century ago, it was established that indole is produced by various bacteria such as *E. coli*, *Vibrio Cholerae*, *Schigella dysenteriae*, and by various species of *Proteus*. However, nothing was known about its precursors or its metabolism. Speculations were made on its formation, such as that tryptophan was converted to indole by step-wise removal of side chain carbons, one by one. However, in 1935, Happold (71) reported that indole was formed enzymatically from tryptophan by tryptophanase. It is now well known that tryptophanase is an enzyme which has pyridoxal phosphate as its coenzyme and catalyzes the formation of indole from tryptophan according to the following equation (72, 73, 74, 75).

Figure 1. Possible Biochemical Pathways for the Formation of Simple Indole Compounds From Tryptophan in Plants.

The pathways indicated with a solid arrow are supported by experimental evidence (70).

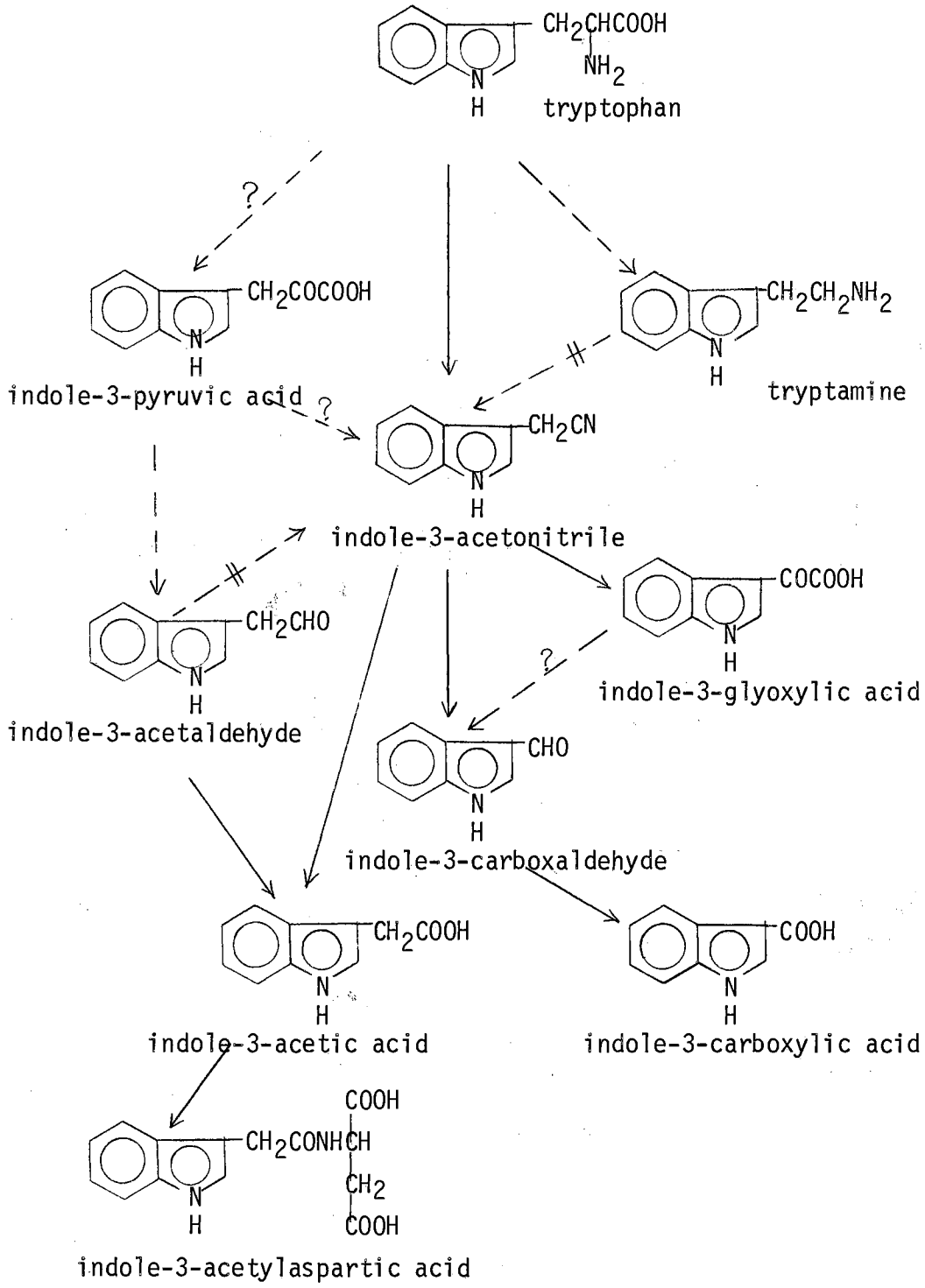

CH2CHCOOH
NH2
tryptophan
?
CH2COCOOH
indole-3-pyruvic acid
CH2CH2NH2
tryptamine
?
CH2CN
indole-3-acetonitrile
CH2CHO
indole-3-acetaldehyde
COCOOH
indole-3-glyoxylic acid
?
CHO
indole-3-carboxaldehyde
CH2COOH
indole-3-acetic acid
COOH
indole-3-carboxylic acid
COOH
CH2CONHCH
CH2
COOH
indole-3-acetylaspartic acid

Figure 2. Possible Pathways for the Biosynthesis of Indoleacetic Acid in Microorganisms (51).

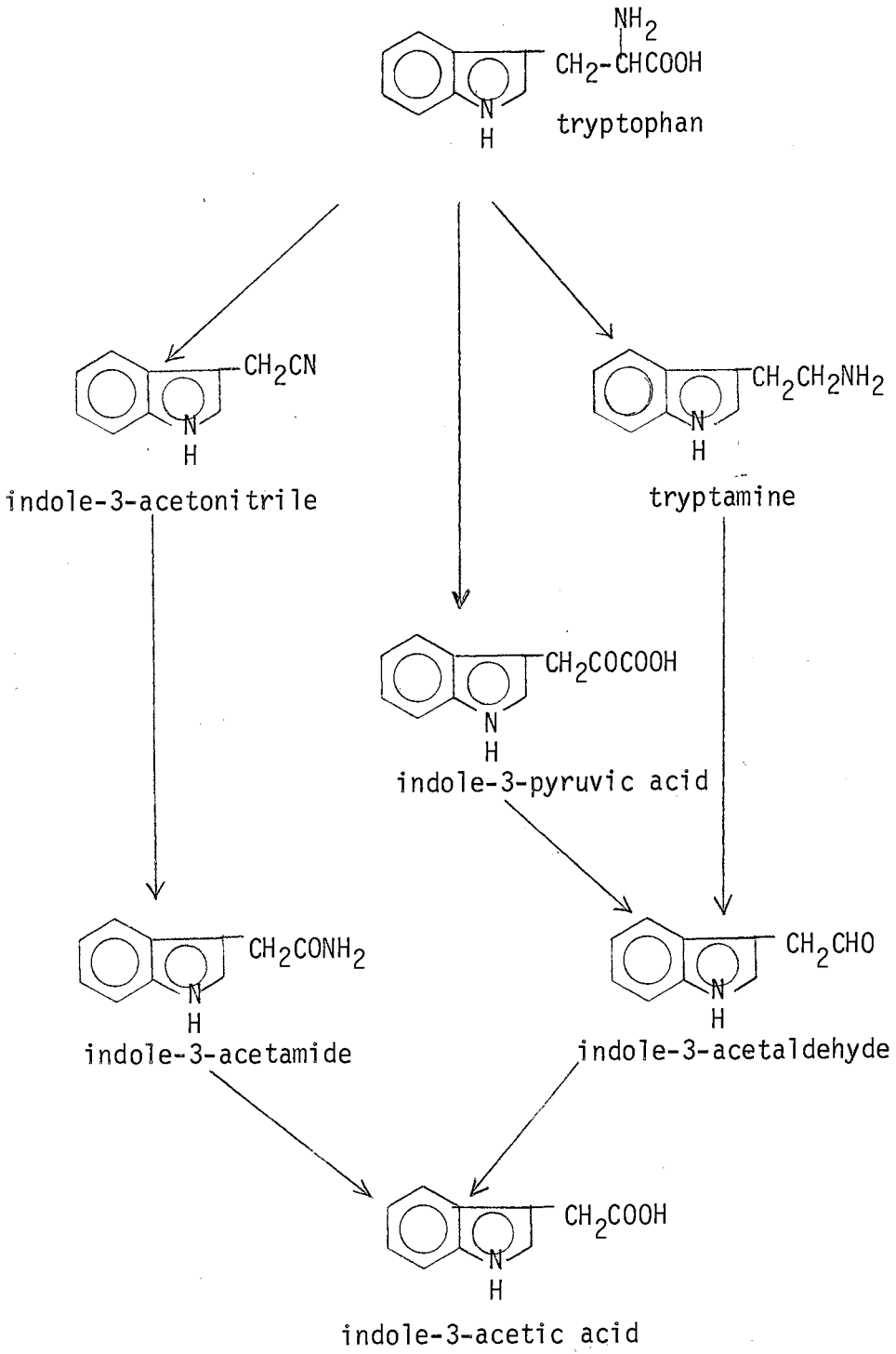
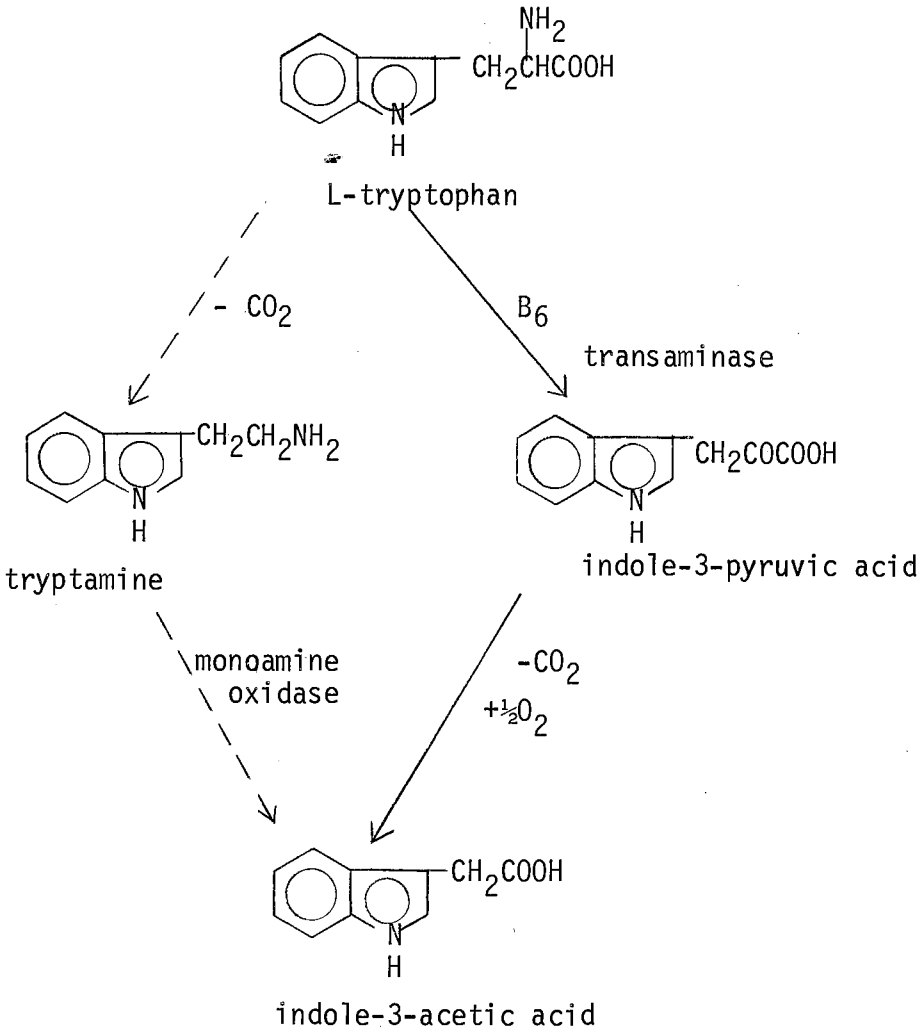
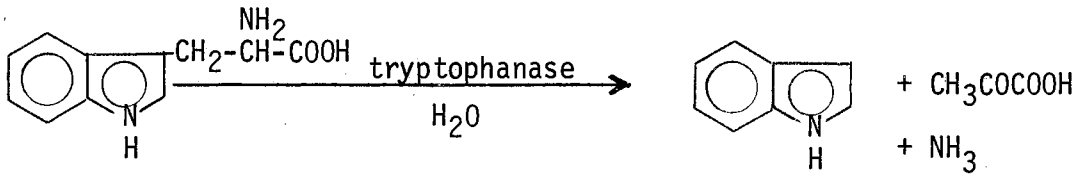

NH2
CH2-CHCOOH
N
H
tryptophan
CH2CN
N
H
indole-3-acetonitrile
CH2CH2NH2
N
H
tryptamine
CH2COCOOH
N
H
indole-3-pyruvic acid
CH2CONH2
N
H
indole-3-acetamide
CH2CHO
N
H
indole-3-acetaldehyde
CH2COOH
N
H
indole-3-acetic acid

Figure 3. Possible Pathways for the Biosynthesis of Indoleacetic Acid in Mammals.

Solid arrows indicate the major pathway (10).


NH2
CH2CHCOOH
N
H
L-tryptophan
- CO2
B6
transaminase
CH2CH2NH2
N
H
tryptamine
CH2COCOOH
N
H
indole-3-pyruvic acid
monoamine
oxidase
-CO2
+½O2
CH2COOH
N
H
indole-3-acetic acid



Recently, Morino and Snell (76) have reported that tryptophanase of E. coli showed absolute requirements for pyridoxal phosphate and for K^+ (or NH_4^+) for activity. At room temperature and pH 8.0, the tryptophanase exists as a tetramer with a molecular weight of 220,000. Reversible dissociation of tryptophanase to a dimer occurs at low temperature or at room temperature in the presence of a very low concentrations of sodium dodecyl sulfate. Ultracentrifugal measurements indicated the presence of four moles of pyridoxal phosphate per mole of holotryptophanase of mol. wt. 220,000.

Further metabolism of indole has been observed in bacteria. Sequential induction studies (77) have indicated that isatin, formylanthranilic acid, anthranilic acid, salicylic acid, and catechol are formed from indole. The fate of indole in mammals is detoxication and excretion in the urine as the salt of the O-sulfate of indoxyl, known as indican (77). Indole produces nausea, headache, and other unpleasant symptoms, whereas indican is harmless (78) and is a normal constituent of urine (79).

Formation of Serotonin

Tryptophan-5-hydroxylase is the enzyme transforming tryptophan into 5-hydroxytryptophan. In 1953 Udenfriend et al. (80) showed that DL-tryptophan-2-^{14}C was converted to labelled 5-hydroxytryptophan by liver

homogenates of rat and guinea pig. But Dalgliesh and Dutton (81, 82) could not confirm the presence of the step, tryptophan ——→ 5-hydroxytryptophan *in vitro* by using an isolated perfused mouse liver. However, there is little doubt that a hydroxylation must occur *in vivo*, for the administration of labelled tryptophan results in the presence of labelled 5-hydroxyindoleacetic acid in the urine (83). This pathway which accounts for only 1% of the administered tryptophan in normal subjects, reaches about 60% in patients with carcinoid, a tumor of the intestinal argentaffin cell (83). This finding suggested that the site where tryptophan was physiologically hydroxylated was in the argentaffin or enterochromaffin cells of the intestine (81, 84).

The presence of tryptophan hydroxylase in brain tissue has been repeatedly demonstrated by work *in vivo* (85, 86, 87) and *in vitro* (88, 89, 90, 91). Peters et al. (92) reported that the distribution of tryptophan hydroxylase in cat brain roughly parallels the distribution of serotonin. The most active areas were the caudate nucleus, septal area, anterior perforating substance, hypothalamus, amygdala and various areas of the midbrain. It has been suggested that tryptophan hydroxylase is the rate-controlling step in the biosynthesis of the neurotransmitter, serotonin, from L-tryptophan (93).

An alternative hypothesis has been considered, namely the possibility that tryptophan is first decarboxylated to yield tryptamine (94, 95, 96) and then tryptamine is hydroxylated to serotonin (97). However, it has been shown that tryptamine cannot be a precursor of serotonin (98).

5-Hydroxytryptophan decarboxylase is the enzyme which converts 5-hydroxytryptophan to 5-hydroxytryptamine (serotonin). When highly

purified, it is quite unstable and sensitive to changes of pH and temperature (99). The enzyme requires the presence of pyridoxal phosphate as cofactor. Pyridoxal phosphate may be replaced by pyridoxamine phosphate, but not by pyridoxine or pyridoxal (100). 5-Hydroxytryptophan decarboxylase is widely distributed in nature. Mammalian kidney and liver are particularly rich in this enzyme which is present in the non-particulate fraction after ultracentrifugation (101). Foetal liver (102) and kidney (103) of rat contain less 5-hydroxytryptophan decarboxylase activity than the respective adult tissues.

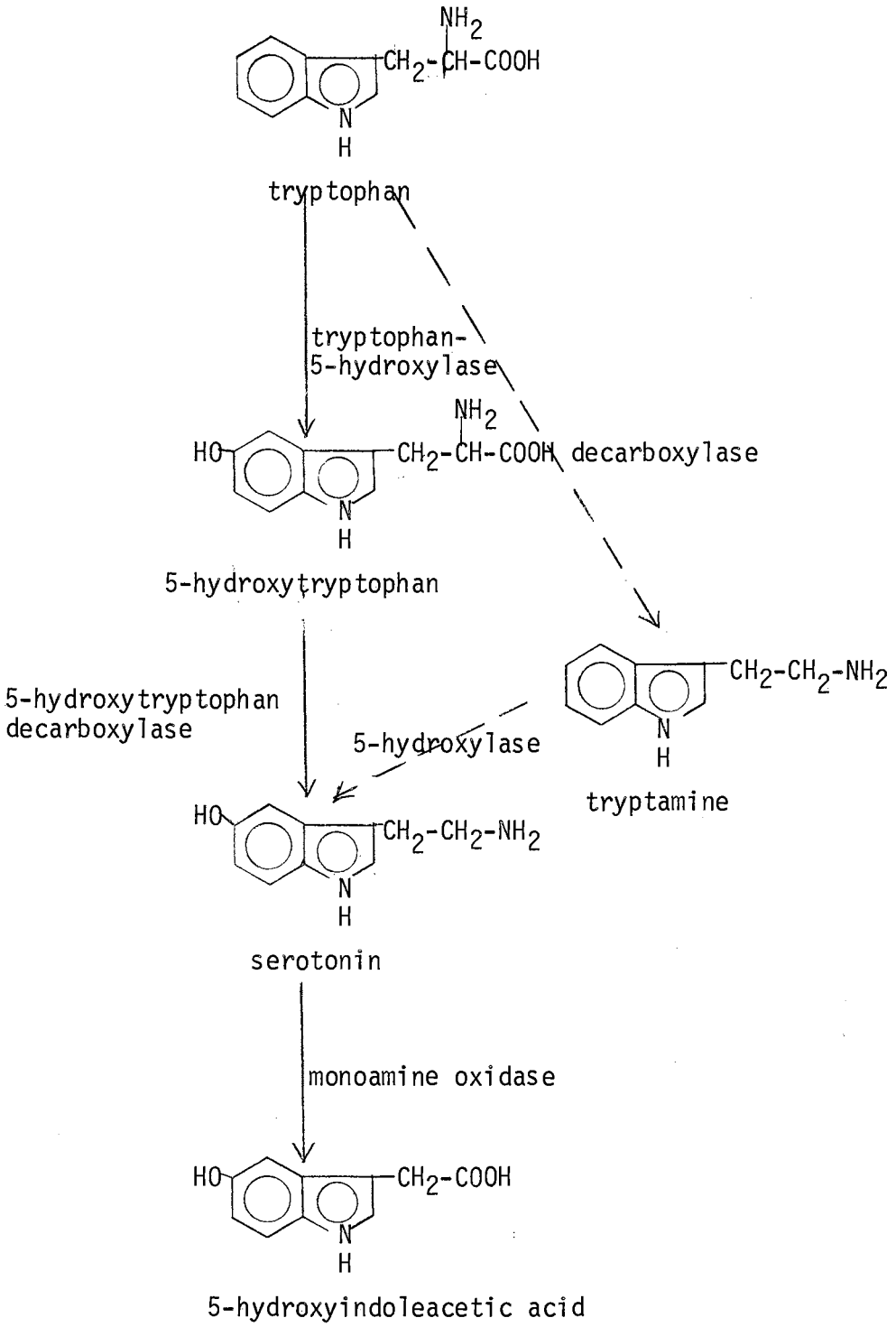
Monoamine oxidase is an enzyme able to transform 5-hydroxytryptamine into 5-hydroxyindoleacetaldehyde which is, in turn, converted to 5-hydroxyindoleacetic acid (104, 105) by an aldehyde dehydrogenase (106, 107). A soluble preparation of monoamine oxidase has been obtained by several methods (107, 108, 109). However, little is known about the structure and the requirements of this enzyme. The metabolic pathway for the formation of serotonin is shown in Figure 4.

Vitamin-B_6-Deficiency and Its Effect on Tryptophan Metabolism

Many species of experimental animals have been shown to develop deficiency symptoms when they are fed diets lacking pyridoxine. At first the changes are minimal, although in young animals a slightly slower growth rate can be detected. Later on there are dermatological changes on the exposed parts (paws and snout), with edema (rat); cessation of growth; elevated systolic blood pressure: appearance of a peculiar gait: degenerative changes in peripheral nerves and posterior roots (pig); lowered threshold to electroshock (rat); microcytic

Figure 4. Possible Biochemical Pathways for the Formation of 5-Hydroxytryptamine (Serotonin) and 5-Hydroxyindoleacetic Acid from Tryptophan in Mammals.

The solid arrows are supported by experimental evidence.


NH2
CH2-CH-COOH
N
H
tryptophan
tryptophan-
5-hydroxylase
HO
CH2-CH-COOH
decarboxylase
5-hydroxytryptophan
5-hydroxytryptophan
decarboxylase
5-hydroxylase
CH2-CH2-NH2
tryptamine
serotonin
monoamine oxidase
CH2-COOH
5-hydroxyindoleacetic acid

anemia (pig, monkey); enlargement of the adrenal gland with marked changes in the microscopic appearance of the cortex; and eventually convulsions (110). All these symptoms respond to pyridoxine administration.

It is well known that a deficiency of vitamin B_6 will alter the normal metabolism of tryptophan in such a manner as to lead to the excretion of large amounts of xanthurenic acid in the urine. Therefore, the appearance of abnormally high amounts of xanthurenic acid in the urine after a loading dose of tryptophan has been generally accepted as a reliable indication of vitamin B_6-deficiency in man and in experimental animals. Vitamin B_6 (pyridoxine) is required as a coenzyme in the catabolism of this amino acid at the stage of 3-hydroxykynurenine, where it plays a role in the scission of the side chain to yield 3-hydroxyanthranilic acid (action of kynureninase). Alternatively, 3-hydroxykynurenine may be converted to the corresponding keto acid under the influence of another pyridoxal phosphate-linked enzyme, hydroxykynurenine transaminase. This keto acid cyclizes spontaneously to form xanthurenic acid. Under conditions of vitamin B_6-deficiency, kynureninase apparently loses its coenzyme more readily than does the hydroxykynurenine transaminase (111), with the result that the balance of tryptophan breakdown is upset and abnormally large amounts of xanthurenic acid are excreted in the urine. Also it has been reported that the vitamin B_6-deficient animals excreted abnormally large quantities of kynurenine, 3-hydroxykynurenine and acetylkynurenine in addition to xanthurenic acid (112, 113, 114).

Tissue levels of serotonin were found to be markedly diminished in vitamin B_6-deficient animals, since vitamin B_6 is a coenzyme for

5-hydroxytryptophan decarboxylase activity (115, 116). It was reported that vitamin b_6-deficiency decreased glycine, L-tryptophan and L-methionine uptake into the cells of animals from a suspending fluid (117).

CHAPTER III

METHODS AND EXPERIMENTS

Animal Experiments

Male rats (Holtzman Co., Wisconsin) weighing 350-400 gm were injected intraperitoneally with D- or L-tryptophan (17 mg/100 gm of bodyweight) along with 10 μc D- or L-tryptophan-methylene-^{14}C. Rats of similar size were injected intraperitoneally with 3.5 mg/100 gm of indoleacetic acid along with 4.8 μc indoleacetic acid-2-^{14}C for each rat. Vitamin B_6-deficiency was obtained by feeding rats weighing approximately 100 gm with a vitamin B_6-deficient diet for 5-15 weeks before use. Vitamin B_6-deficient rats were injected with the same concentration of tryptophan or indoleacetic acid as normal rats. Experiments utilizing germ-free rats were conducted by Dr. N. Raica at Fitzsimons General Hospital, U. S. Army Medical Research and Nutrition Laboratory, Denver, Colorado. The urine of animals from these experiments (urines of germ-free rats injected with D- or L-tryptophan-methylene-^{14}C) were lyophilized and shipped to the author for analysis.

The experimental animals were transferred into an all-glass metabolism cage immediately after injection. Urine was collected from each rat for 24 hours in a vessel containing a few drops of 0.5 N acetic acid and surrounded by dry ice. The urines were filtered through sintered glass, lyophilized and stored at -20° C. The exhalation of $^{14}CO_2$ from experimental germ-free rats was measured.

Chemical Synthesis of Indoleaceturic Acid

Since indoleaceturic acid (indoleacetylglycine) is not available from a commercial source, it was synthesized according to the method of Wieland and Hörlein (118).

Indoleacetic acid 1.76 gm and 1.4 ml triethylamine were dissolved in 30 ml of absolute tetrahydrofuran. The mixture was cooled to 0° C, and 0.96 ml of ethyl chloroformate was added dropwise with shaking. After 5 min, the glycine solution (750 mg glycine dissolved in 8 ml of 1.25 M NaOH) was added to the reaction mixture. After standing at 0° C for 30 min until it decolorized due to the decomposition of the anhydride, the solution was brought to room temperature with shaking. The reaction mixture was evaporated under vacuum to remove the volatile components, and was acidified with 2 N HCl until it became cloudy (pH 3-4). A precipitated syrup became solid after scratching with a glass rod. The solid was filtered and washed with water to free it from indoleacetic acid contamination. The purified solid was dissolved in warm water and formed a clear supernatant and a brown oily precipitate; the brown oily precipitate was removed by centrifugation and the clear supernatant was kept in the cold room until white needle crystals formed.

The complete separation of crystallized indoleaceturic acid from indoleacetic acid was accomplished by fractional crystallization. The crystals were dissolved in a minimum volume of ethyl acetate, after which, petroleum ether (40-80° C) was added dropwise until the solution became cloudy. The cloudy solution was stored in the cold room to precipitate crystals. This procedure was repeated twice.

The purified crystals showed a M. P. of 86-88° C. The purity was

confirmed by paper chromatography (119, 120). After hydrolysis with 0.5 N HCl at 100°C for one hour the substance showed a molar ratio of indoleacetic acid and glycine of 1:1.

Column Chromatography

Since a satisfactory column chromatographic separation of indole metabolites of tryptophan was not available, several types of columns, such as silica-gel (121), Amberlite IR-120 (pyridine form) (122), Sephadex G-25 (123), Sephadex G-10, DEAE-Sephadex A-25, Dowex-1 (formate), Florisil and DEAE-cellulose, were investigated. A dual column of DEAE-cellulose chromatography system using triethylamine-formate (TEA-F) buffer as solvent was developed for this research.

DEAE-cellulose was poured with stirring into a beaker containing about 20 volumes (volume liquid/dry weight exchanger) of 0.5 N HCl and left for 30 min. The supernatant liquid was decanted and the solid washed with deionized water until the supernatant solution was around pH 4.0. This supernatant was decanted and the resulting exchanger was poured into a beaker containing 5 volumes (volume liquid/swollen exchanger) of 0.5 N NaOH with gentle stirring and left for 30 min. The supernatant liquid was decanted and the solid was washed with deionized water until the supernatant liquid was around pH 9.0. The DEAE-cellulose was then packed into a large column (5 x 75 cm) and eluted with deionized water until the effluent was neutral. This exchanger was used for DEAE-cellulose (amine-form) column chromatography.

The DEAE-cellulose (amine form) in a large column (5 x 75 cm) was washed with 10 volumes of 1 M sodium formate and the excess sodium formate was washed out with deionized water. Then the column was

treated with 0.001 M TEA-F of pH 4.0 until the exchanger was equilibrated. This exchanger was used for the first DEAE-cellulose column chromatography (formate form).

Several authentic tryptophan metabolites were loaded on a 1.2 x 30 cm column of DEAE-cellulose (formate). The column was then eluted with 80 ml of pH 4.0, 0.001 M TEA-F (2 ml/fraction), followed by a linear gradient from 0.001 M to 0.1 M of the same buffer solution (4 ml/fraction) in 250 ml:250 ml.

The chromatogram is shown in Figure 5. The first two peaks (approximately 30 ml-60 ml) in Figure 5 contain several components. The fractions from the two peaks were combined and lyophilized. The lyophilized material was dissolved in about 5-10 ml water and loaded on a second column of DEAE-cellulose (amine-form). This column was eluted with 140 ml pH 8.0, 0.001 M TEA-F (2 ml/fraction), followed by a linear gradient from 0.001 M to 0.05 M of the same buffer solution in 200 ml : 200 ml (4 ml/fraction). The column chromatogram is shown in Figure 6. Both columns were run in the cold at $\cong 5^{o}C$. The recovery of the authentic compounds was more than 95% from both DEAE-cellulose columns.

Column chromatography for the separation of compounds in the crude urine samples was carried out using the same procedures as for the authentic compounds. To assure complete elution of all radioactive metabolites, a linear gradient from 0.1 M to 0.3 M of TEA-F, pH 4.0 (4 ml/fraction) in 200 ml : 200 ml was used on the DEAE-cellulose-formate column after the linear gradient from 0.001 M to 0.1 M TEA-F.

Paper Chromatography

Descending paper chromatography following the procedures of

Figure 5. Separation of Tryptophan Metabolites on DEAE-Cellulose (Formate) Column.

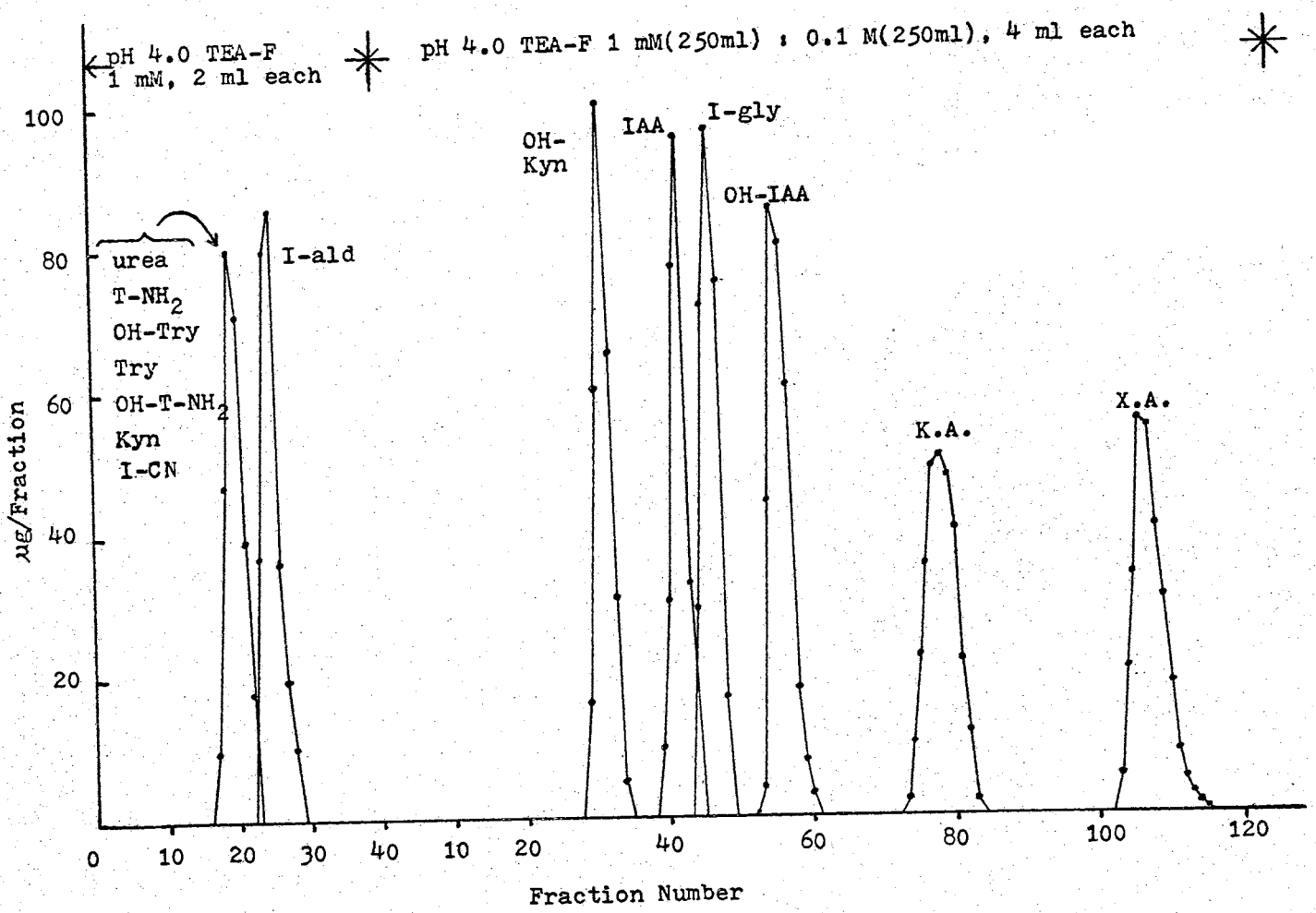
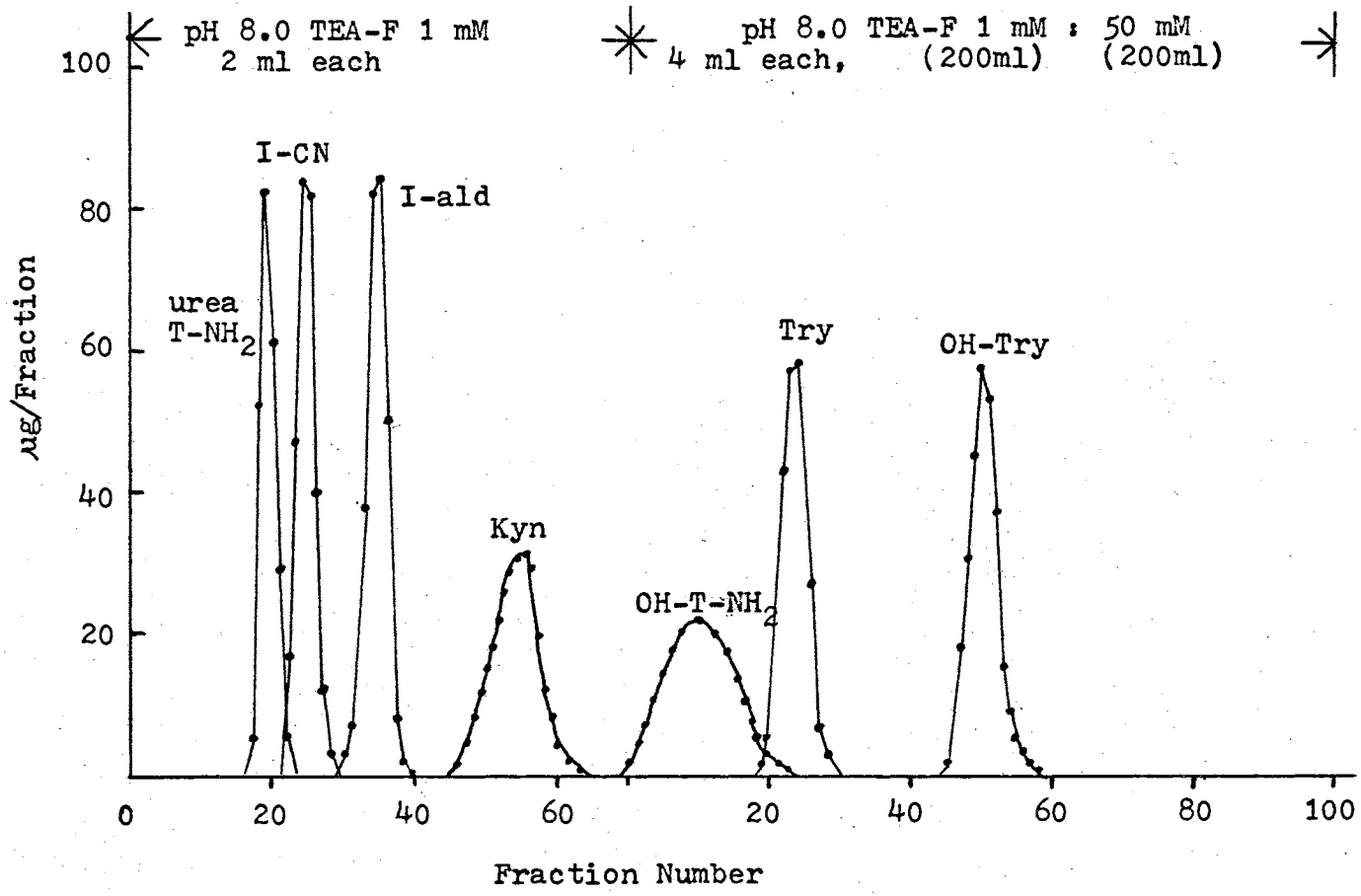

pH 4.0 TEA-F
1 mM, 2 ml each
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml), 4 ml each
urea
T-NH2
OH-Try
Try
OH-T-NH2
Kyn
I-CN
I-ald
OH-
Kyn
IAA
I-gly
OH-IAA
K.A.
X.A.
μg/Fraction
Fraction Number

Figure 6. Separation of Tryptophan Metabolites on DEAE-Cellulose Column.


pH 8.0 TEA-F 1 mM
2 ml each
pH 8.0 TEA-F 1 mM : 50 mM
4 ml each, (200ml) (200ml)
I-CN
I-ald
urea
T-NH2
Kyn
OH-T-NH2
Try
OH-Try
µg/Fraction
Fraction Number
0
20
40
60
80
100

Dalgliesh (119) and Armstrong et al. (120) was employed as one of the techniques for isolation and identification of radioactive metabolites of tryptophan-methylene-^{14}C and indoleacetic acid-2-^{14}C. The location of various compounds was detected by chemical methods, such as Ehrlich's reagent (120), 2,4-dinitrophenylhydrazine (120), ninhydrin reagent (0.2% ninhydrin in 95% ethanol), by UV fluorescence and by detection of radioactivity on a Nuclear Chicago 4π strip counter.

Colorimetry and Fluorimetry

Quantitative and qualitative determination of the authentic compounds and the corresponding radioactive metabolites was carried out as follows.

Indoleacetic acid and indoleaceturic acid were determined by the Gordon-Weber modification of the Salkowski's reagent (124) and Ehrlich's reagent (125). To one ml of aqueous solution, two ml of Salkowski's reagent (0.01 M ferric chloride in 35% of perchloric acid) was added with shaking. After standing for 50 min absorbance due to indoleacetic acid and indoleaceturic acid was determined at 530 mμ and 550 mμ, respectively, on a Coleman Junior Spectrophotometer. In the other method, to one ml of aqueous solution, two ml of Ehrlich's reagent (10 gm of p-dimethylaminobenzaldehyde dissolved in 100 ml of conc hydrochloric acid) was added with shaking. The resulting violet color was read immediately at 550 mμ (both indoleacetic acid and indoleaceturic acid).

Indole-3-carboxaldehyde was determined by the method of Stutz (64). The sample solution was evaporated to dryness using a rotary evaporator. Absolute ethanol (2.5 ml) was added and to this ethanol

solution was added 0.25 ml of 2,4-dinitrophenylhydrazine reagent (100 mg of 2,4-dinitrophenylhydrazine in 100 ml of 6 N HCl). The colored solution was read immediately at 550 mμ.

Tryptophan, indoleacetonitrile, kynurenine and N-acetylkynurenine were determined by the Chen-Gholson modification of the N-1-naphthylethylenediamine dihydrochloride method (126). To a 2 ml aqueous solution were successively added: 0.2 ml of 2.5% sodium nitrite and 0.2 ml of oxidizing agent (mixture of 1 volume 60% perchloric acid and 4 volumes conc sulfuric acid, the mixture diluted to double volume with water), 4 min later 0.2 ml of 10% ammonium sulfamate were added followed 4 min later by 0.2 ml of 0.25% N-1-napthylethylenediamine dihydrochloride. After standing 20 min (indoleacetonitrile) or one hour (tryptophan, kynurenine and N-acetylkynurenine) at room temperature, the colored solutions were read at 550 mμ (indoleacetonitrile, kynurenine and N-acetylkynurenine) or 555 mμ (tryptophan).

Tryptamine, serotonin and 5-hydroxytryptophan were determined by Ehrlich's reagent (125) as described in the indoleacetic acid determination.

5-Hydroxindoleacetic acid was determined by the method of Ratliff (127). To 1 ml of aqueous sample solution was successively added: 0.5 ml of 1-nitroso-2-naphthol (0.1% solution in 95% ethanol) and 0.5 ml nitrous acid reagent (freshly prepared 2.5% sodium nitrite in water), 20 min later 5 ml of ethylene chloride was added, the mixture stoppered and shaken for 1 min. After standing several min, the upper layer was pipetted off and was read at 540 mμ.

3-Hydroxykynurenine and xanthurenic acid were determined according to the method of Coppini et al. (128). To 2 ml of aqueous sample

solution, 0.6 ml of diazotized sulfanilic acid (0.5% p-diazobenzene sulfonic acid in 2% hydrochloric acid, mixed before using with an equal volume of 0.5% sodium nitrite in water) and 0.12 ml of pyridine were added. The absorbancy due to xanthurenic acid was read immediately at 510 mμ. The 3-hydroxykynurenine sample was read after 1 hour at 450 mμ. Xanthurenic acid was alternatively measured by adding an equal volume of 50% NaOH to the aqueous sample solution. After centrifuging the sample was determined fluorometrically at 535 mμ (excitation 370 mμ) using an Aminco-Bowman Spectrophotofluorometer.

Kynurenic acid was determined according to the method of Benassi et al. (122). To 1.5 ml aqueous sample solution, 1 ml of conc sulfuric acid was added slowly while cooling with ice. After 15 min. the sample was read fluorometrically at 333 mμ excitation wavelength and 440 mμ emission wavelength.

Other Methods

Each fraction eluted from the DEAE-cellulose column was monitored for radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer by pipetting a 0.1 ml aliquot into 10 ml of scintillation fluid. The scintillation fluid was composed of 600 ml toluene, 400 ml ethanol, 4.0 gm of 2,5-diphenyl-oxazole and 0.2 gm of 1,4-bis [2-(5-phenyloxazolyl)]-benzene (129). The quantitative measurement for each metabolite was based primarily on the radioactivity determination. Each radioactive peak was measured for its absorption spectrum by using a Beckman DB Spectrophotometer. Subsequently, each radioactive peak was lyophilized and dissolved in a minimum volume of deionized water or acetone for its identification.

The new metabolite, indole-3-carboxaldehyde was crystalized by the method of Shaw and Rysen (130). The 2,4-dinitrophenylhydrazone was prepared (131) and the melting point was measured. Ultraviolet spectra at different pH's were measured by using a Beckman DB Spectrophotometer. Infrared spectra were determined using a Perkin-Elmer 457 Grating Infrared Spectrophotometer in KBr pellets prepared with a micro-sampling kit. Mass spectra were determined with the prototype (132) of the LKB-900 gas chromatograph-mass spectrometer using a direct inlet probe. Melting points were determined with a Kofler Micro M. P. apparatus.

Chemicals

D- and L-tryptophan-methylene-^{14}C and indole-3-acetic acid-2-^{14}C were obtained from Nuclear Chicago Corp.; L-tryptophan, indole-3-acetic acid, kynurenic acid and xanthurenic acid from Sigma Chemical Co.; tryptamine hydrochloride, D-5-hydroxytryptophan, indole-3-carboxaldehyde and 5-hydroxyindoleacetic acid from Aldrich Chemical Co.; D-tryptophan and L-kynurenine sulfate from Calbiochem Laboratories. 3-Hydroxy-DL-kynurenine was a gift from Dr. L. M. Henderson of the University of Minnesota, Minneapolis, Minnesota. Serotonin creatinine sulfate and indole-3-acetonitrile were purchased from K & K Laboratories. DEAE-cellulose (Cellex-D) and Dowex-1 were purchased from Bio-Rad Laboratories. DEAE-Sephadex A-25, Sephadex G-10 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals Inc. Amberlite CG-120, silicic acid (100 mesh) were purchased from Mallinckrodt Chemical Works. Florisil (60-100 mesh) and p-dimethylaminobenzaldehyde were purchased from Fisher Scientific Co. 2,4-Dinitrophenylhydrazine,

N-1-naphthylethylenediamine dihydrochloride, ammonium sulfamate and 1-nitroso-2-naphthol were obtained from Eastman Kodak Co. Vitamin B-complex test diet was obtained from Nutritional Biochemicals Corp. All other chemicals were of reagent grade and were obtained from local supply houses.

CHAPTER IV

RESULTS

Identification of the New Metabolite

During the course of this study, a previously unreported urinary metabolite of tryptophan, indole-3-carboxaldehyde (I-ald), was isolated from the urines of normal, vitamin B_6-deficient, germ-free and germ-free vitamin B_6-deficient rats following the intraperitoneal injection of D-tryptophan-methylene-^{14}C. The isolation of this new metabolite from DEAE-cellulose columns is shown in Figures 11, 13, 15, and 18.

The new metabolite was eluted in the 50 ml to 80 ml fractions of 0.001 M effluent from the second column (DEAE-cellulose-amine form). The compound was tentatively identified as I-ald by ultraviolet spectrum, paper chromatography in 4 different solvent systems (119, 120), peak coincidence between radioactivity and colorimetric determination of the 2,4-dinitrophenylhydrazone, and by co-column chromatography with authentic I-ald.

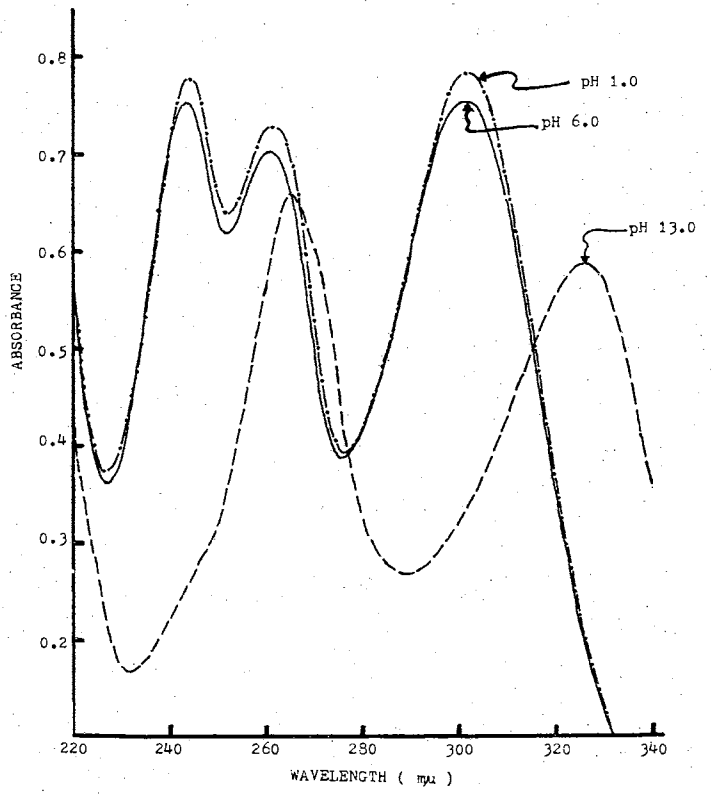
In order to further confirm this unknown compound as I-ald, the fractions containing this metabolite were combined then lyophilized and crystallized. The melting points of I-ald and its 2,4-dinitrophenylhydrazone derivative were found to be 194-8°C and 273-8°C, respectively. A mixed melting point with an authentic sample of I-ald showed no depression.

Figure 7 shows a comparison of the absorption spectra at different

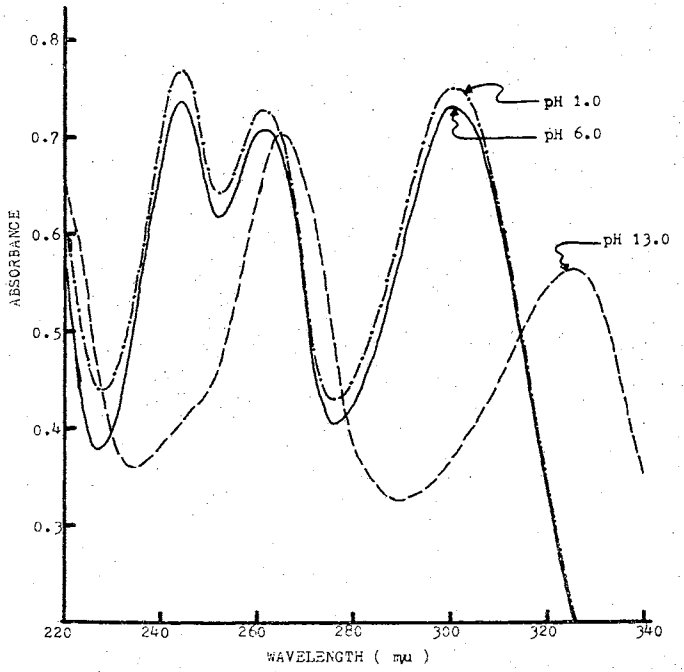
Figure 7. Ultraviolet Sprectra of Authentic Indole-3-carboxaldehyde and the Material Isolated from Rat Urine.

———— = compound dissolved in H_2O; —·—·—·—·— = in 0.1 N HCl; — — — — — = in 0.1 N NaOH.

ULTRAVIOLET SPECTRA OF INDOLE-3-CARBOXALDEHYDE



ULTRAVIOLET SPECTRA OF UNKNOWN COMPOUND



pH values of authentic I-ald and the urinary I-ald in the ultraviolet region. The concentration of the authentic compound was 4 μM, and from the calculation based on radioactivity, the concentration of this new metabolite was 3.9 μM. The change in the UV spectrum at pH 13.0 was interpreted (67) as being due to the following conversion:

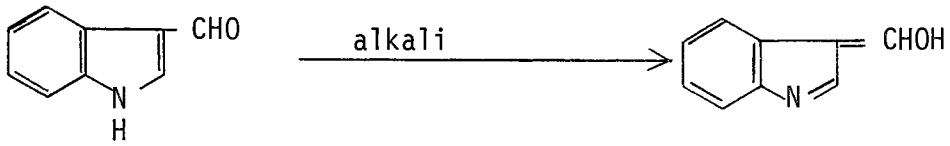


Figure 8 shows the infrared absorption spectra for the urinary compound and authentic I-ald. The N-H stretching is characterized by a broad band between 3120 and 2900 CM^{-1} in both spectra. The carbonyl stretching frequency occurs at 1631 CM^{-1}. The similarity of these spectra is evident.

The mass spectral fragmentation patterns for the unknown and authentic compounds are in excellent agreement as shown in Figure 9. Both show a base peak at 144, a parent peak at 145 and an indole fragment at 116. An impurity in the purchased compound at mass 149 was observed, but it did not contribute significantly to the total ion production.

From Table I, it is evident that I-ald is one of the major metabolites of D-tryptophan in the rat. This metabolite was not detected in the urine of rats injected with L-tryptophan.

Figure 8. Infrared Spectra of Authentic Indole-3-Carboxaldehyde and the Material Isolated from Rat Urine.

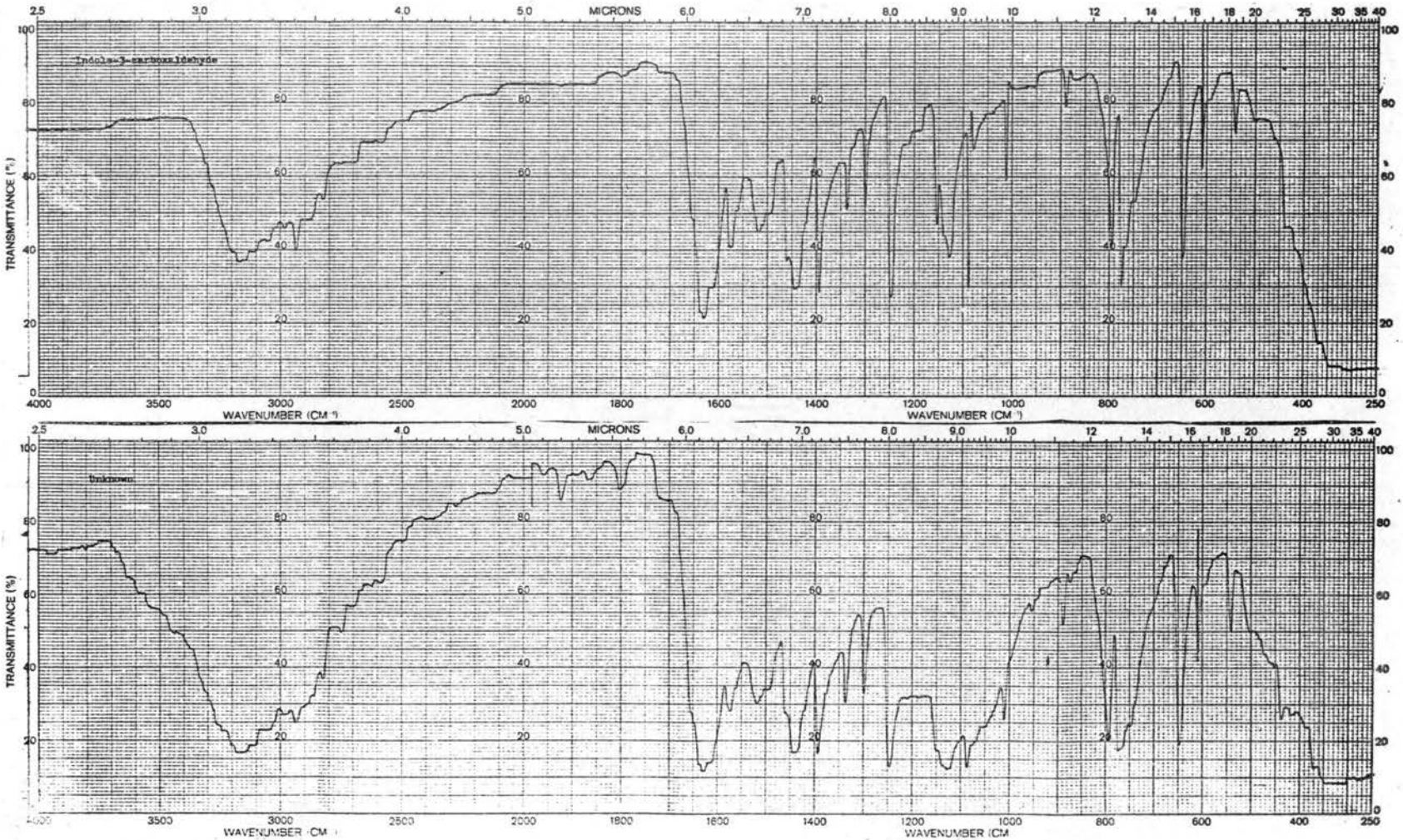

Indole-3-carboxaldehyde
MICRONS
TRANSMITTANCE (%)
WAVENUMBER (CM⁻¹)
Unknown
MICRONS
TRANSMITTANCE (%)
WAVENUMBER (CM⁻¹)

Figure 9. Mass Spectra of Authentic Indole-3-Carboxaldehyde and the Material Isolated from Rat Urine.

The spectra were computer plotted from tabular intensity data (133).

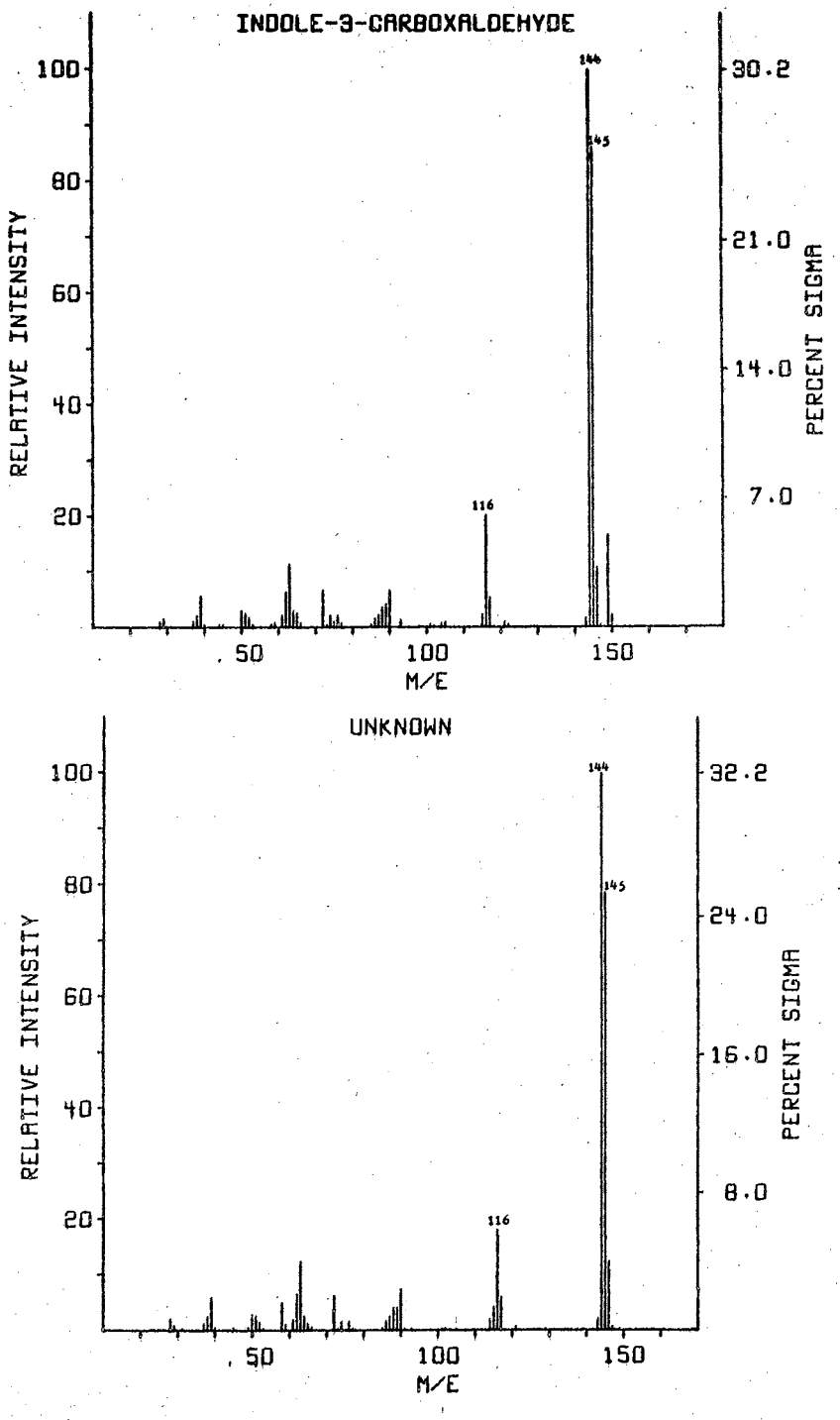

INDOLE-3-CARBOXALDEHYDE
RELATIVE INTENSITY
100
80
60
40
20
144
145
116
30.2
21.0
14.0
7.0
PERCENT SIGMA
50
100
150
M/E
UNKNOWN
RELATIVE INTENSITY
100
80
60
40
20
144
145
116
32.2
24.0
16.0
8.0
PERCENT SIGMA
50
100
150
M/E

TABLE I

DISTRIBUTION OF RADIOACTIVITY AMONG METABOLITES IN 24-HOUR URINE SAMPLES OF NORMAL AND B_6-DEFICIENT RATS FOLLOWING THE INTRAPERITONEAL INJECTION OF 17 MG/100G D-TRYPTOPHAN-METHYLENE-^{14}C (% OF DOSE)

	Germ-free		Normal	
Metabolites	B_6-fed	B_6-def	B_6-fed	B_6-def
Co_2 output	5.8	7.7		
^{14}C % excreted in urine	38.3	59.0	39.8	28.5
Urea	2.6	2.0	7.5	0.9
Tryptophan	2.1	8.6	13.5	14.0
Indoleacetic acid	7.8	5.5	3.9	1.9
Indoleaceturic acid	5.0	8.8	2.4	3.9
Indole-3-carboxaldehyde	1.9	6.1	5.4	1.7
Kynurenine	1.0	trace	trace	1.2
3-Hydroxykynurenine	0	2.5	0	0
Kynurenic acid	2.3	3.9	0	0.8
Xanthurenic acid	0	0	0	0
X. A. conjugate	0	0.8	0	0
N-Acetylkynurenine	2.0	2.3	0.6	0.9
N-Acetyl-3-hydroxy-kynurenine	1.7	1.5	0.4	0.6
Tryptamine	0	1.9	0	0.5
	GFD1e 1.9	B_6GFD8 1.1		
	GFD6 1.7	B_6GFD9 trace		

Urinary Metabolites of D- and L-Tryptophan-Methylene-^{14}C in Germ-Free and Normal Rats

Metabolites of D-Tryptophan in B_6-Fed Animals

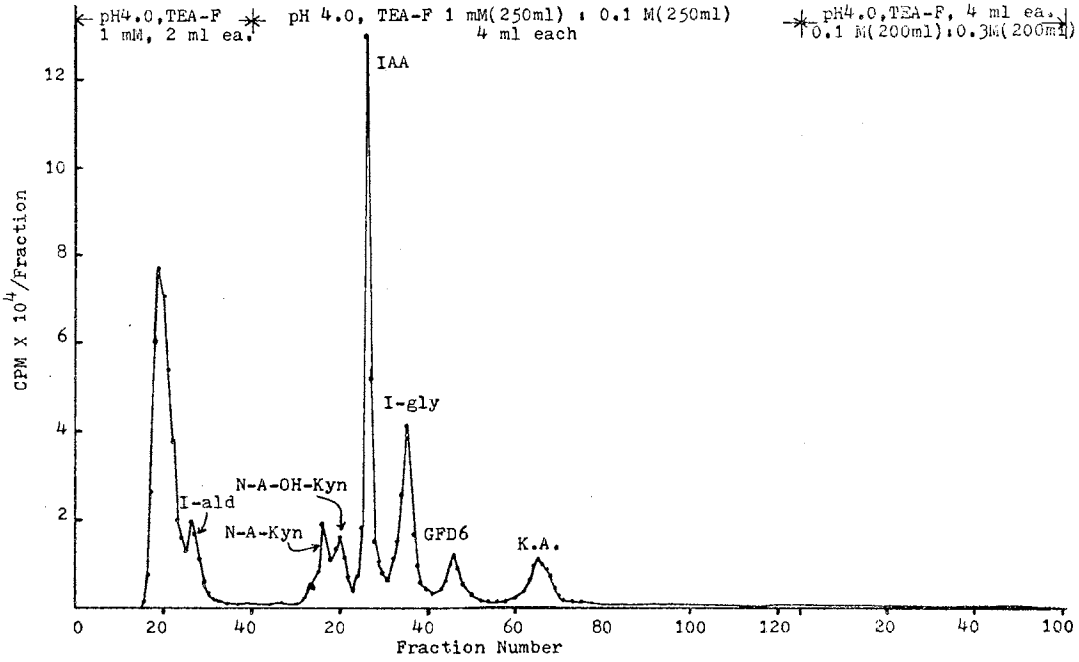
The radioactive metabolites isolated from the urine of germ-free and normal rats injected with D-tryptophan-methylene-^{14}C are shown in Figures 10 and 11 and Figures 12 and 13, respectively.

In the germ-free rat.--There are at least 12 radioactive metabolites that have been isolated in this experiment (Figures 10 and 11). Nine of these metabolites have been identified, and are listed as follows: urea, I-ald, kynurenine (Kyn), tryptophan (Try), N-acetyl-kynurenine (N-A-Kyn), N-acetyl-3-hydroxykynurenine (N-A-OH-Kyn), IAA, indoleaceturic acid (I-gly) and kynurenic acid (K. A.). The quantitative data are shown in Table I. IAA (7.8% of total dose) and its glycine conjugate, I-gly (5.0% of total dose) are the major urinary metabolites. The new metabolite, I-ald, was isolated but in less quantity than that isolated from the urine of the normal rat. N-A-Kyn and N-A-OH-Kyn were observed as the metabolites of D- and L-tryptophan either in B_6-fed or B_6-deficient animals. Three metabolites, GFDle, GFDlf and GFD6 have not been identified as yet.

In the normal rat.--Seven known metabolites have been isolated and identified as urea, Try, N-A-Kyn, N-A-OH-Kyn, IAA, I-gly, a trace of Kyn and a new metabolite identified as I-ald (Figures 12 and 13). The kynurenine was separated by paper chromatography in BuOH-HAc-H_2O (4:1:5 by vol) and 20% (W/V) KCl, from the radio active peak of I-ald shown in Figure 13. Urea, Try, IAA and I-gly are the major metabolites in this experiment. The quantitative data are shown in Table I.

Figure 10. Separation of Urinary Tryptophan Metabolites of Germ-Free Rat Injected with D-Tryptophan-Methylene^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 11. Rechromatography of Combined Fractions 14-35 From the pH 4.0 Column (Figure 10) on the pH 8.0 DEAE-Cellulose Column.


pH4.0,TEA-F 1 mM, 2 ml ea.
pH 4.0, TEA-F 1 mM(250ml) : 0.1 M(250ml) 4 ml each
pH4.0,TEA-F, 4 ml ea. 0.1 M(200ml):0.3M(200ml)
IAA
I-gly
N-A-OH-Kyn
I-ald
N-A-Kyn
GFD6
K.A.
CPM X 10^4/Fraction
Fraction Number

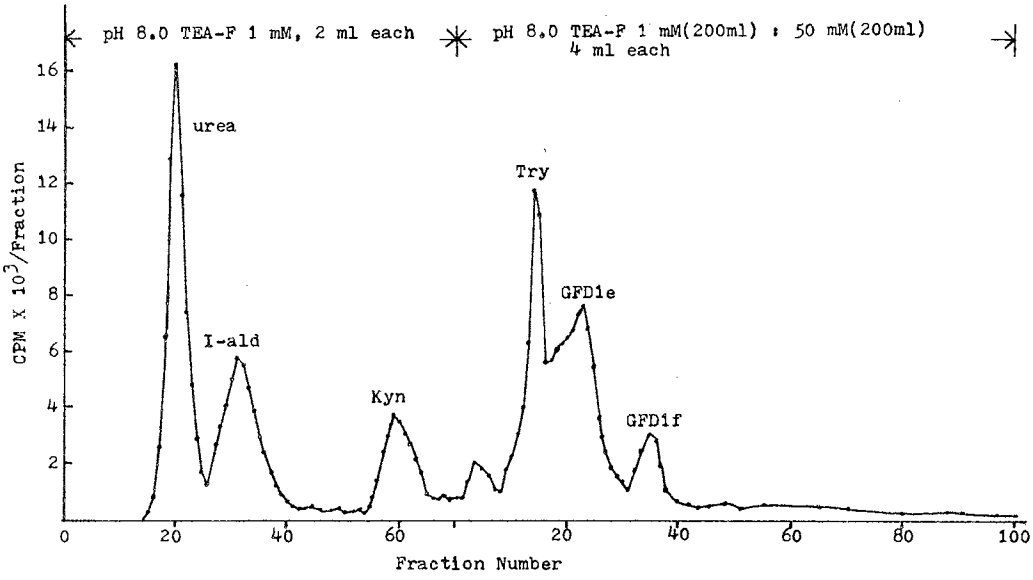
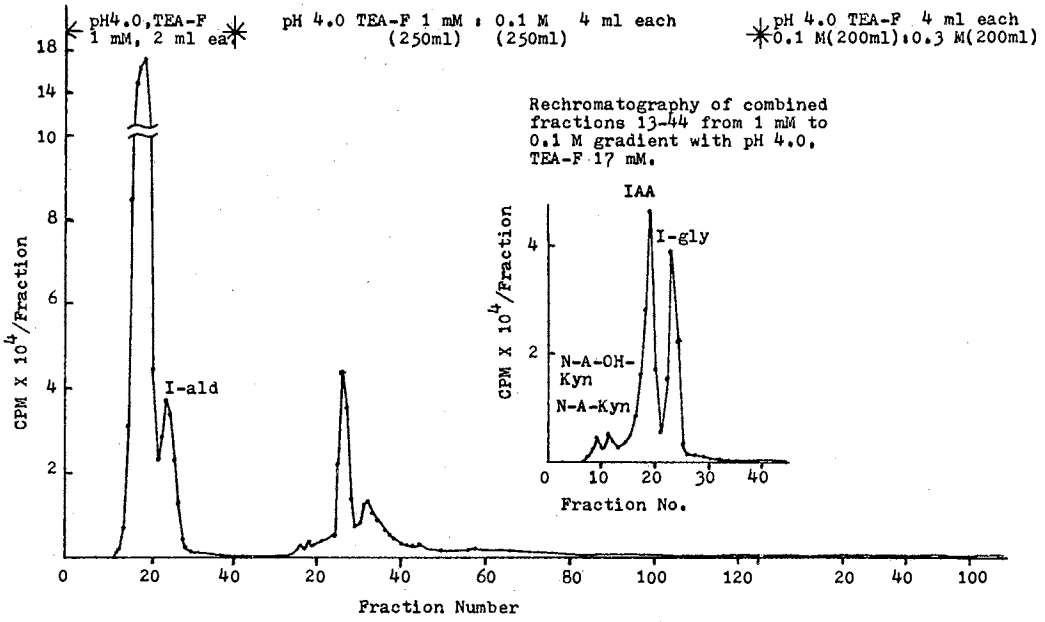
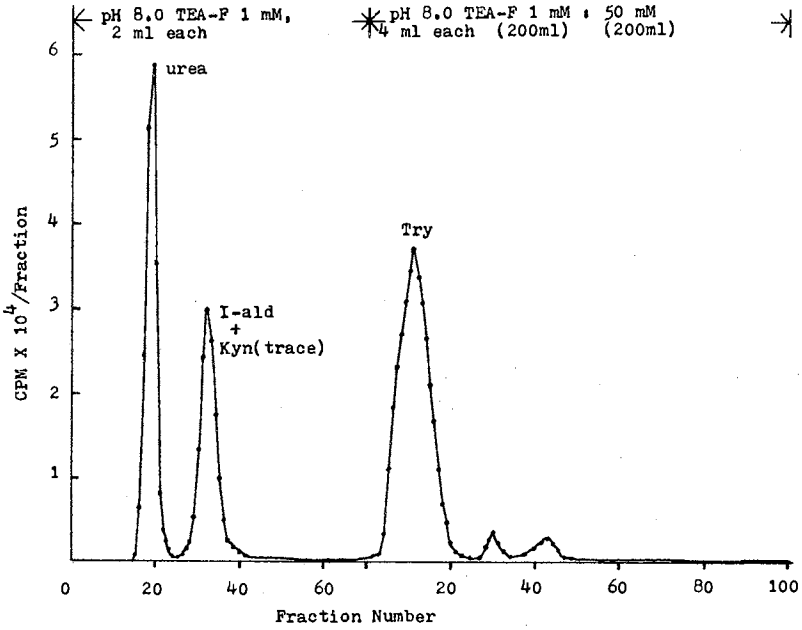

pH 8.0 TEA-F 1 mM, 2 ml each
pH 8.0 TEA-F 1 mM(200ml) : 50 mM(200ml) 4 ml each
urea
Try
GFD1e
I-ald
Kyn
GFD1f
CPM X 10^3/Fraction
Fraction Number

Figure 12. Separation of Urinary Tryptophan Metabolites of Normal Rat Injected with D-Tryptophane-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 13. Rechromatography of Combined Fractions 12-34 from the pH 4.0 Column (Figure 12) on the pH 8.0 DEAE-Cellulose Column.

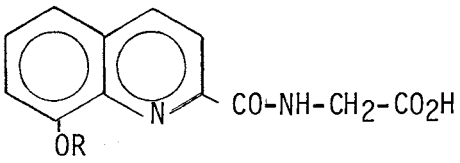

pH4.0,TEA-F 1 mM, 2 ml ea
pH 4.0 TEA-F 1 mM ; 0.1 M 4 ml each
(250ml) (250ml)
pH 4.0 TEA-F 4 ml each
0.1 M(200ml);0.3 M(200ml)
Rechromatography of combined fractions 13-44 from 1 mM to 0.1 M gradient with pH 4.0, TEA-F 17 mM.
IAA
I-gly
N-A-OH-Kyn
N-A-Kyn
I-ald
CPM X 10^4/Fraction
Fraction No.
Fraction Number


pH 8.0 TEA-F 1 mM, 2 ml each
pH 8.0 TEA-F 1 mM ; 50 mM
4 ml each (200ml) (200ml)
urea
I-ald + Kyn(trace)
Try
CPM X 10^4/Fraction
Fraction Number

Metabolites of D-Tryptophan in B_6-Deficient Animals

In the germ-free rat.--At least 14 metabolites were isolated as shown in Figures 14 and 15. Eleven of the metabolites were identified as urea, tryptamine (T-HH_2), I-ald, Try, N-A-Kyn, N-A-OH-Kyn, IAA, I-gly, 3-hydroxykynurenine (OH-Kyn), K. A. and a trace of Kyn. T-NH_2 was isolated by the previously described paper chromatography method from the first peak of Figure 15. The substance, B_6GFD10 (unknown I), shows a UV spectrum similar to that of xanthurenic acid (X. A.). After partial hydrolysis (hydrolysis in 0.5 N HCl at 100°C for one hour) of this substance, a X. A. spot was detected by paper chromatography. The conjugate compound of X. A. was also isolated from the urines of L-tryptophan injected germ-free and normal rats under B_6-depletion. The properties of this X. A. conjugate compound and authentic X. A. are given in Table II. A comparison of the UV spectra of the authentic X. A., urinary X. A. and the Unknown I (X. A. conjugate compound) is shown in Figure 16.

In 1960, Baglioni et al.(134) isolated two metabolites from the urines of rats after administration of X. A. They identified them as X. A. conjugate compounds. The proposed structures are:



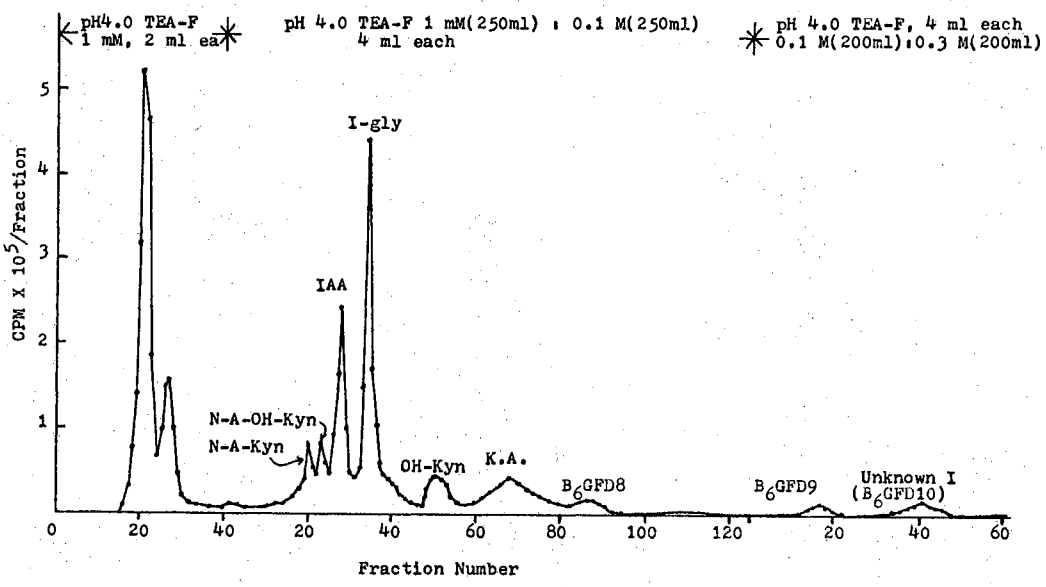
I: R = SO_3H

II: R = $C_6H_9O_6$

Substance I is a compound formed by X. A., glycine and sulfate. Its Rf is 0.14 in BuOH-HAc-H_2O (4:1:5 by vol) and 0.47 in 20% (W/V) KCl. Substance II is a compound formed by X. A., glycine and glucuronic acid with an Rf of 0.09 in BuOH-HAc-H_2O and 0.53 in 20% KCl.

Figure 14. Separation of Urinary Tryptophan Metabolites of B_6-Deficient Germ-Free Rat Injected with D-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 15. Rechromatography of Combined Fractions 16-34 from the pH 4.0 Column (Figure 14) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F
1 mM, 2 ml ea
pH 4.0 TEA-F 1 mM(250ml) ; 0.1 M(250ml)
4 ml each
pH 4.0 TEA-F, 4 ml each
0.1 M(200ml);0.3 M(200ml)
CPM X 10^5/Fraction
I-gly
IAA
N-A-OH-Kyn
N-A-Kyn
OH-Kyn
K.A.
B6GFD8
B6GFD9
Unknown I
(B6GFD10)
Fraction Number

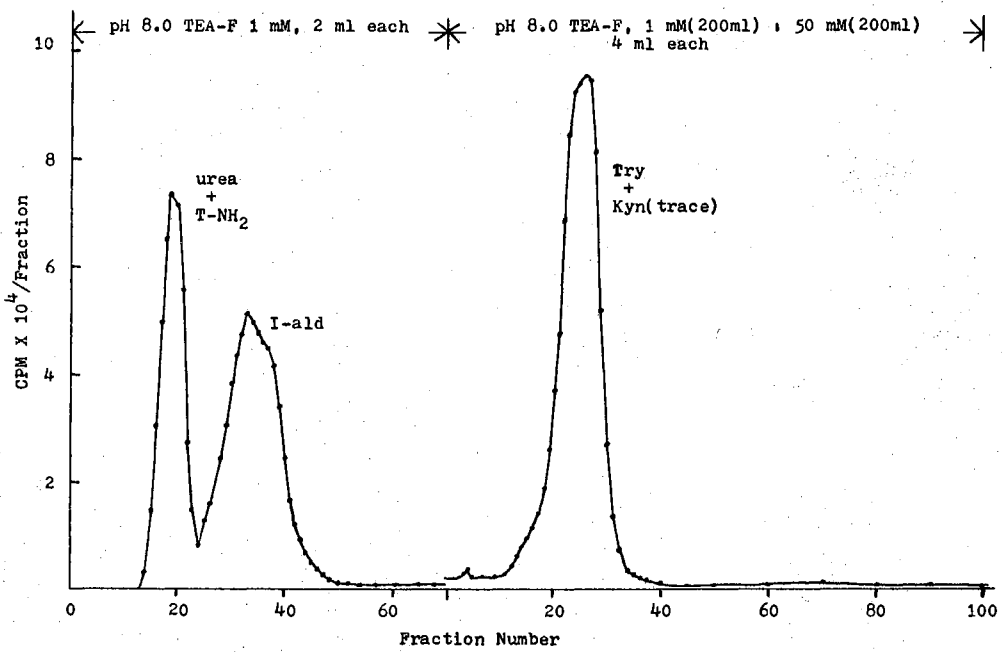

pH 8.0 TEA-F 1 mM, 2 ml each
pH 8.0 TEA-F, 1 mM(200ml) ; 50 mM(200ml)
4 ml each
CPM X 10^4/Fraction
urea
+
T-NH2
I-ald
Try
+
Kyn(trace)
Fraction Number

TABLE II

PROPERTIES OF URINARY CONJUGATE OF XANTHURENIC ACID AND AUTHENTIC XANTHURENIC ACID

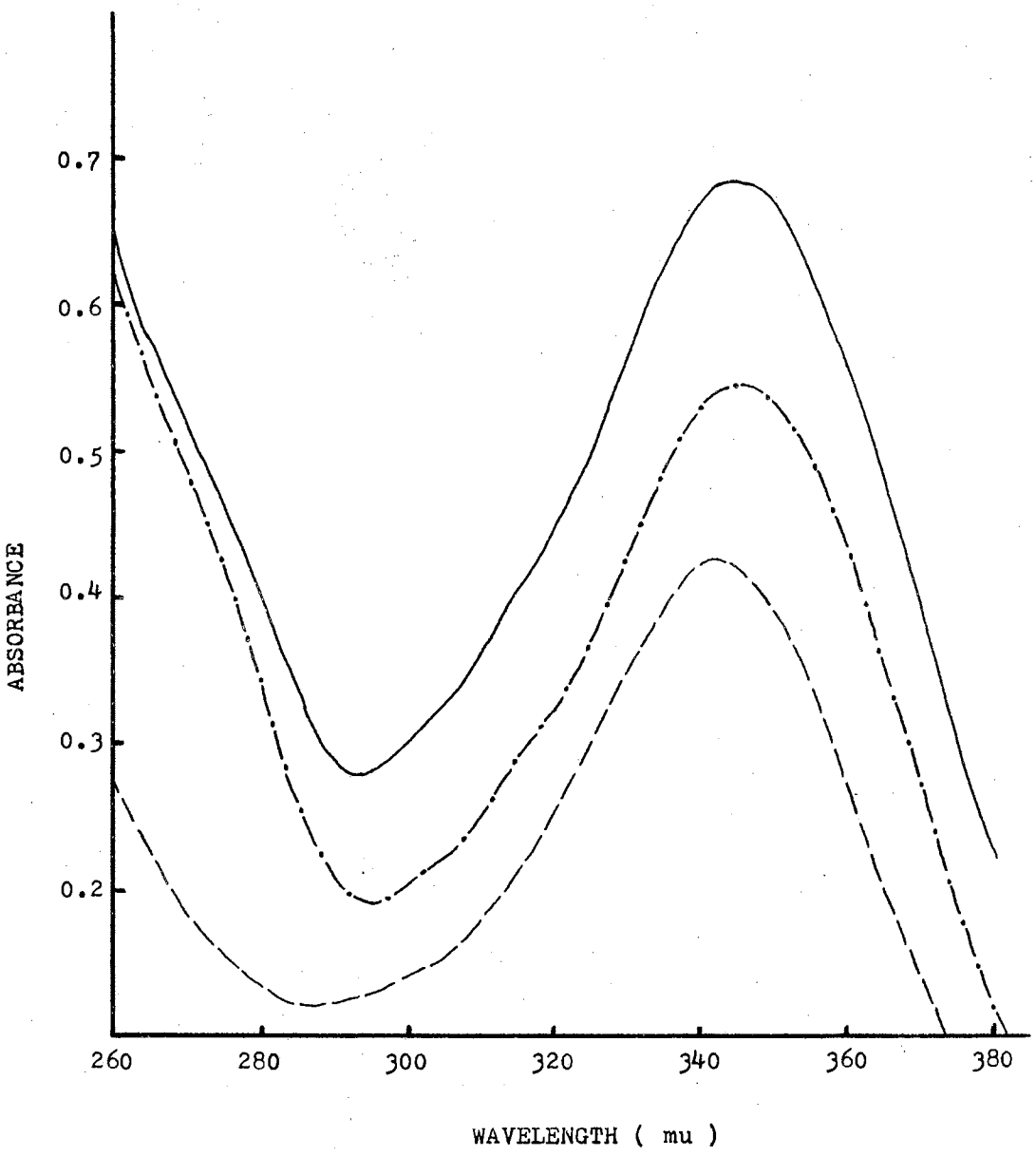
	Unknown I* (Before hydrolysis)	Unknown I (After hydrolysis)	X. A.
Rf in BuOH-HAc-H_2O[#] (4:1:5 by vol)	0.10-0.13	0.56-0.58	0.56-0.59
Rf in 20% (w/v) KCl	0.48-0.54	0.21-0.24	0.20-0.23
Ehrlich's reagent test	Negative	?	
Ninhydrin test	Negative	?	
Fluorescence	Blue	Blue	Blue

*Urinary conjugate of xanthurenic acid.

[#]Dalgliesh (119) used the organic phase of BuOH-HAc-H_2O(4:1:5) for a solvent system.

Figure 16. Absorption Spectra of Authentic Xanthurenic Acid, Urinary Xanthurenic Acid and Urinary Conjugate Compound of Xanthurenic Acid in pH 4.0 TEA-F Buffer.

——— = authentic xanthurenic acid; ——·—·—— = urinary xanthurenic acid; — — — — = urinary conjugate compound of xanthurenic acid.


0.7
0.6
0.5
0.4
0.3
0.2
ABSORBANCE
260
280
300
320
340
360
380
WAVELENGTH (mu)

The isolated compound, Unknown I (B_6GFD10 of Figure 14, B_6GFL8 of Figure 23, and B_6LN7 of Figure 25) shows similarities to both Substance I and II. The Rf value of this Unknown I in the BuOH-HAc-H_2O system is 0.10-0.13 and 0.48-0.54 in 20% KCl. Since it was only partially hydrolyzed and the quantity in the urine was small, it was difficult to detect sulfate, glycine and glucuronate residues in the hydrolysate. Further studies should be made of these residues.

In this experiment, I-ald, IAA and I-gly are observed as the major metabolites. T-NH_2 and urea overlapped in the effluent from both of the columns.

In the normal rat.--In this experiment, ten metabolites were isolated and identified as urea, T-NH_2, Kyn, Try, N-A-Kyn, N-A-OH-Kyn, IAA, I-gly, I-ald and K. A. as shown in Figures 17 and 18 and Table I.

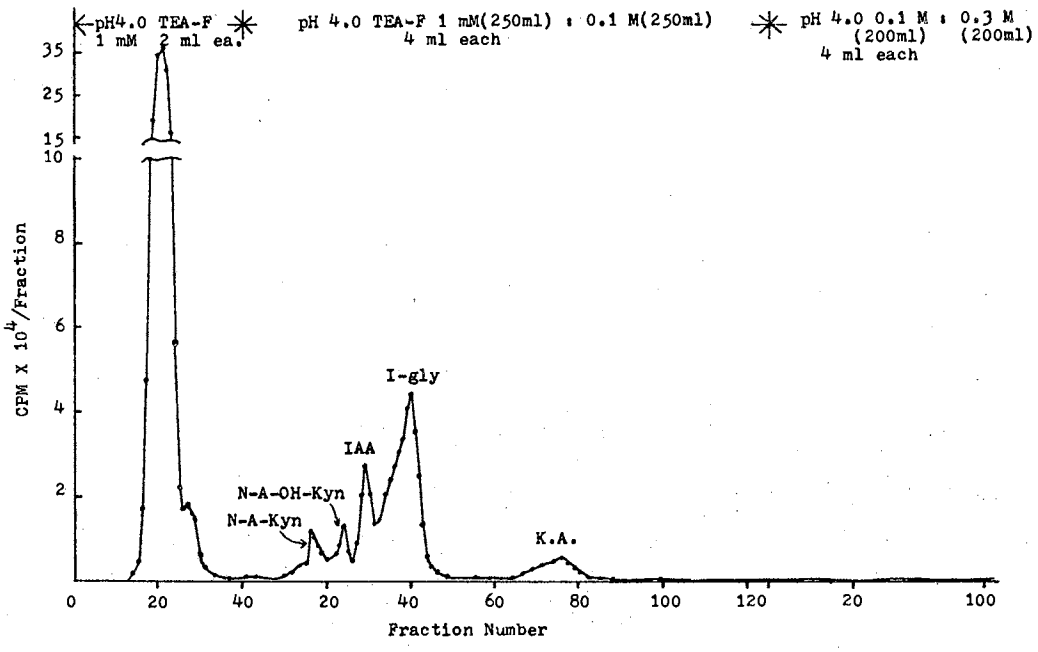
Of interest is the fact that the excretion of I-gly by the vitamin B_6-deficient animal, after the administration of D-tryptophan, is higher than that of IAA; the reverse is observed in the B_6-fed animal. Moreover, I-ald is confirmed as one of the major metabolites of D-tryptophan excreted by the rat. No vitamin B_6 effect on the formation of this indole compound was observed.

Metabolites of L-Tryptophan in B_6-Fed Animals

In the germ-free rat.--Six metabolites were isolated and identified as urea, K. A., N-A-Kyn, N-A-OH-Kyn, IAA and I-gly (Figures 19 and 20 and Table III). The excretion of IAA and I-gly was much less in this run as compared to that from the animals injected with D-tryptophan. I-ald was not detected in this experiment. The quantitative data are summarized in Table III.

Figure 17. Separation of Urinary Tryptophan Metabolites of B_6-Deficient Rat Injected with D-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 18. Rechromatography of Combined Fractions 14-35 from the pH 4.0 Column (Figure 17) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F 1 mM 2 ml ea.
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml) 4 ml each
pH 4.0 0.1 M : 0.3 M (200ml) (200ml) 4 ml each
CPM X 10^4/Fraction
I-gly
IAA
N-A-OH-Kyn
N-A-Kyn
K.A.
Fraction Number

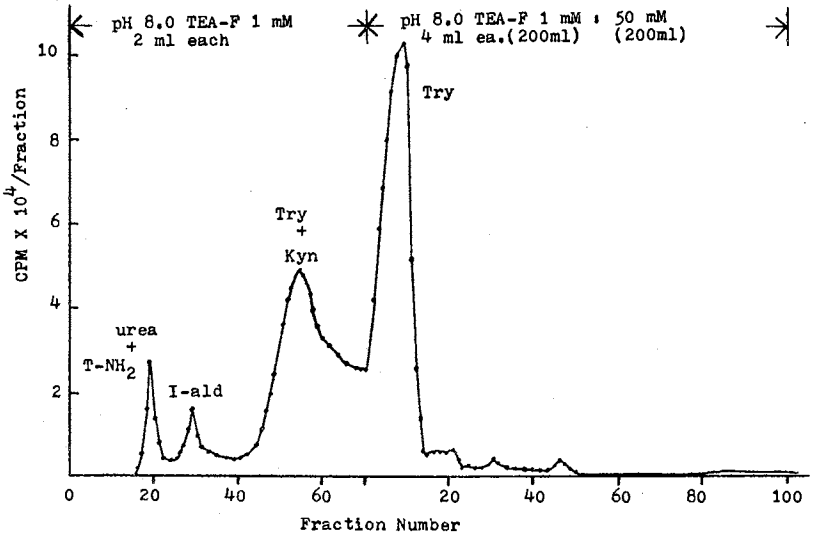
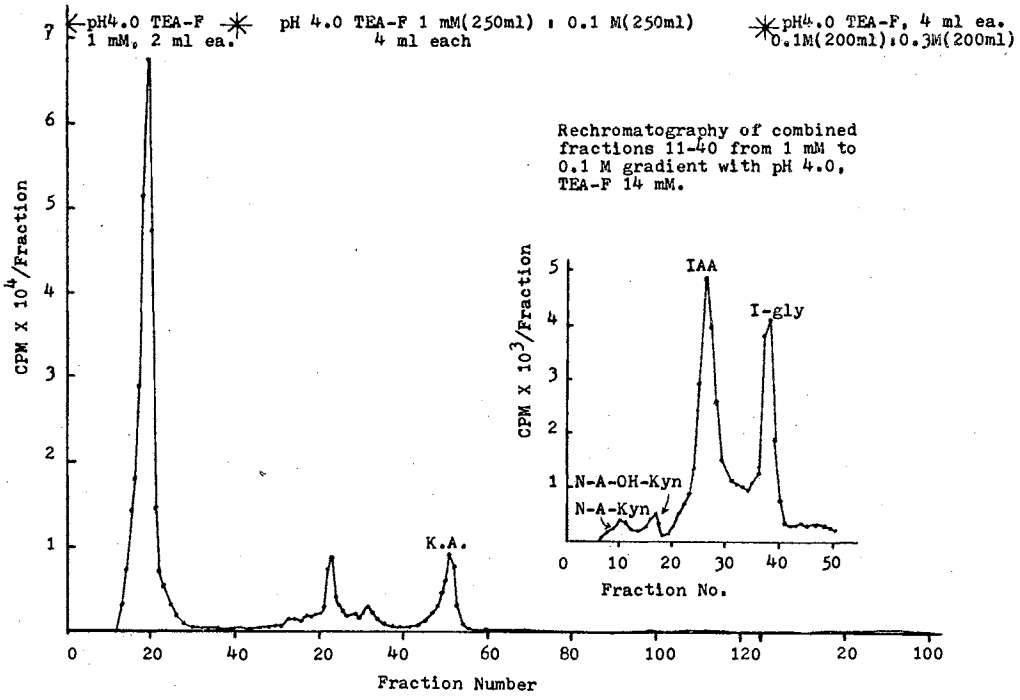

pH 8.0 TEA-F 1 mM 2 ml each
pH 8.0 TEA-F 1 mM : 50 mM 4 ml ea.(200ml) (200ml)
CPM X 10^4/Fraction
Try
Try + Kyn
urea + T-NH2
I-ald
Fraction Number

Figure 19. Separation of Urinary Tryptophan Metabolites of Germ-Free Rat Injected with L-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 20. Rechromatography of Combined Fractions 13-35 from the pH 4.0 Column (Figure 19) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F 1 mM, 2 ml ea.
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml) 4 ml each
pH4.0 TEA-F, 4 ml ea. 0.1M(200ml):0.3M(200ml)
CPM X 10^4/Fraction
K.A.
Fraction Number
Rechromatography of combined fractions 11-40 from 1 mM to 0.1 M gradient with pH 4.0, TEA-F 14 mM.
CPM X 10^3/Fraction
IAA
I-gly
N-A-OH-Kyn
N-A-Kyn
Fraction No.

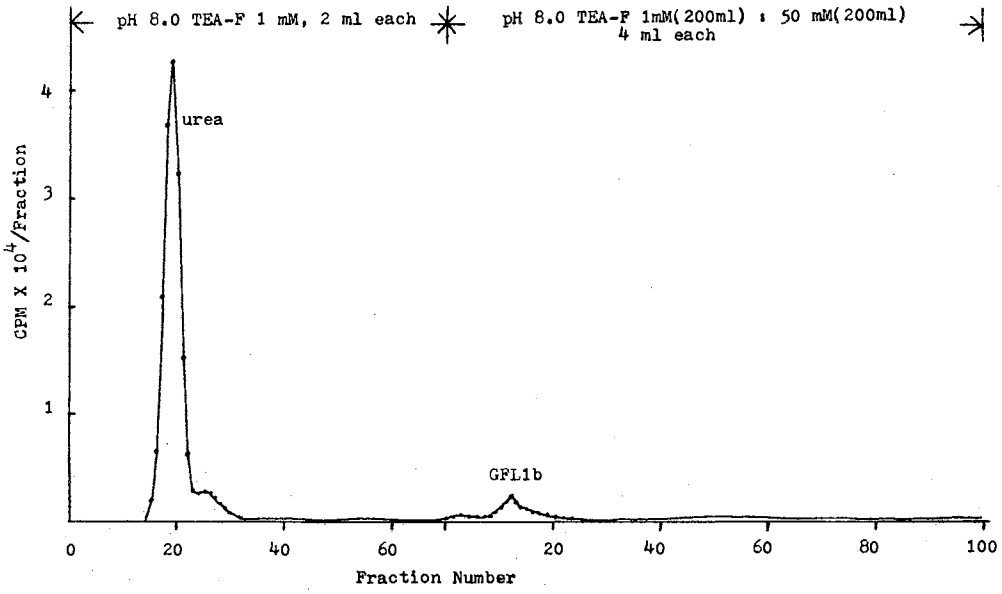

pH 8.0 TEA-F 1 mM, 2 ml each
pH 8.0 TEA-F 1mM(200ml) : 50 mM(200ml) 4 ml each
CPM X 10^4/Fraction
urea
GPL1b
Fraction Number

TABLE III

DISTRIBUTION OF RADIOACTIVITY AMONG METABOLITES IN 24-HOUR URINE SAMPLES OF NORMAL AND B_6-DEFICIENT RATS FOLLOWING THE INTRAPERITONEAL INJECTION OF 17 MG/100G L-TRYPTOPHAN-METHYLENE-^{14}C (% OF DOSE)

	Germ-free		Normal	
Metabolites	B_6-fed	B_6-def	B_6-fed	B_6-def
CO_2 output	40.6	23.1		
^{14}C % excreted in urine	8.0	36.1	10.5	36.1
Urea	3.9	1.7	4.0	0.7
Tryptophan	?	0.7	?	1.2
Indoleacetic acid	0.6	0	0.6	0
Indoleaceturic acid	0.3	0	0.3	0
Indole-3-carboxaldehyde	0	0	0	0
Kynurenine	0	trace	0	1.4
3-Hydroxykynurenine	0	8.4	0	3.0
Kynurenic acid	0.7	1.9	trace	2.0
Xanthurenic acid	0	7.5	0	4.1
X. A. conjugate	0	0.7	0	1.5
N-Acetyl-kynurenine	0.1	2.2	1.0	1.5
N-Acetyl-3-hydroxy-kynurenine	0.1	2.1	0.7	2.8
	GFL1b 0.1	B_6GFL1c 0.8	LN1b 0.9	B_6LN1d 1.2
		B_6GFL7 0.8		
		B_6GFL9 0.8		

In the normal rat.--The urinary metabolites of this experiment are the same as those from the germ-free rat. However, only a trace of K. A. was observed in this experiment. Again, a conspicuous amount of N-A-Kyn and N-A-OH-Kyn was observed as compared to that in the germ-free animal. The separation pattern of the urinary radioactive metabolites is shown in Figures 21 and 22.

Metabolites of L-Tryptophan in B_6-Deficient Animals

A remarkable difference between the metabolism of B_6-fed and B_6-deficient animals after administration of L-tryptophan was observed.

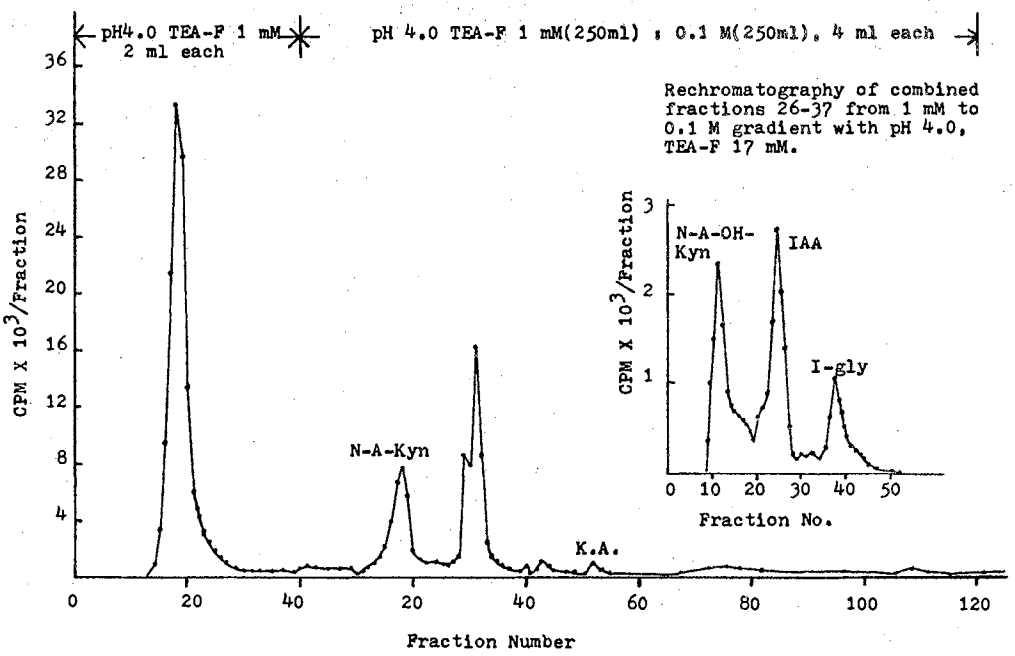
In the germ-free rat.--A large quantity of OH-Kyn and X. A. was isolated. The separation pattern in the dual column chromatography system is shown in Figures 23 and 24. The X. A. conjugate compound (B_6GFL8 of Figure 23, Unknown I) was also observed. The excretion of N-A-Kyn and N-A-OH-Kyn is strikingly larger than that from B_6-fed animals after administration of L-tryptophan. The absence of IAA and I-gly is worthy of note. Several metabolites, including B_6GFL1c of Figure 24, B_6GFL7,8,9 of Figure 23, have not been identified as yet. The properties of B_6GFL7 and B_6GFL9 are given in Table IV.

The properties of B_6GFL7 are the same as those of B_6GFD9 (Figure 14) which was isolated from the urine of the germ-free rat injected with D-tryptophan. The migration properties of the metabolites, B_6GFL7 (B_6GFD9) and B_6GFL9, on paper chromatography are distinctly different from those which have been reported so far for the metabolites of tryptophan and the metabolites of X. A. and quinaldic acid (134, 135, 136).

In the normal rat.--A similarity in the metabolism of the germ-free

Figure 21. Separation of Urinary Tryptophan Metabolites of Normal Rat Injected with L-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 22. Rechromatography of Combined Fractions 14-30 from the pH 4.0 Column (Figure 21) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F 1 mM
2 ml each
pH 4.0 TEA-F 1 mM(250ml) ; 0.1 M(250ml), 4 ml each
Rechromatography of combined fractions 26-37 from 1 mM to 0.1 M gradient with pH 4.0, TEA-F 17 mM.
CPM X 10³/Fraction
N-A-Kyn
K.A.
N-A-OH-Kyn
IAA
I-gly
Fraction No.
Fraction Number

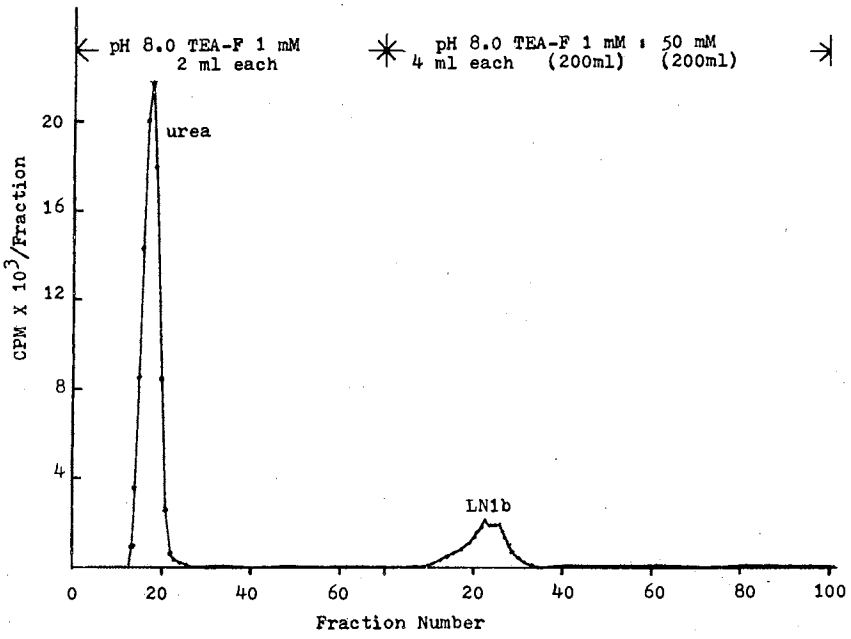
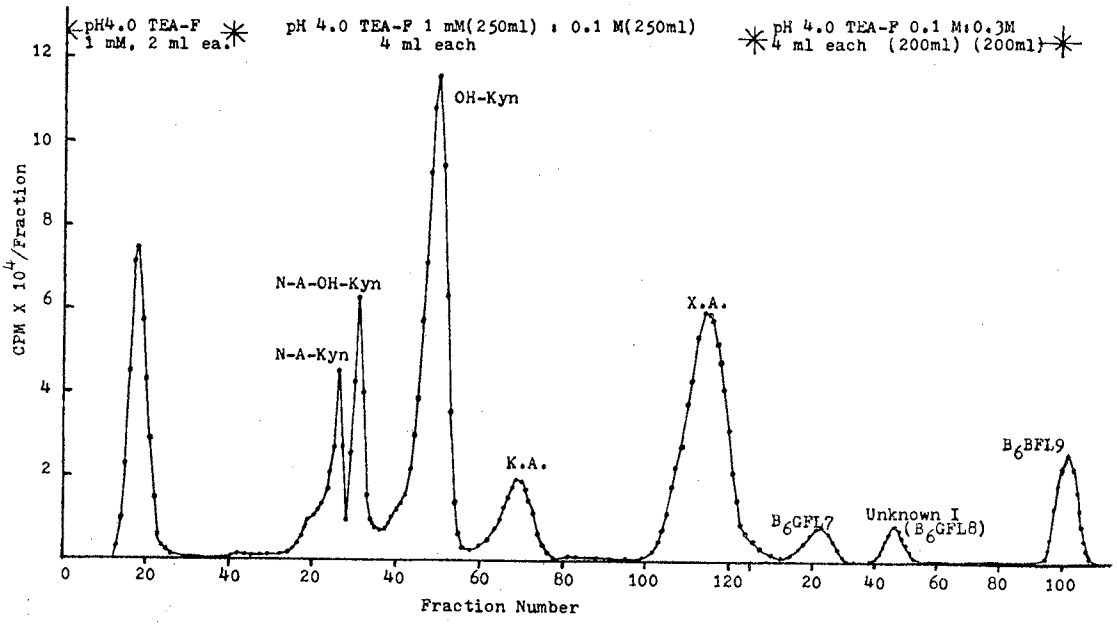

pH 8.0 TEA-F 1 mM
2 ml each
pH 8.0 TEA-F 1 mM ; 50 mM
4 ml each (200ml) (200ml)
CPM X 10³/Fraction
urea
LN1b
Fraction Number

Figure 23. Separation of Urinary Tryptophan Metabolites of B_6-Deficient Germ-Free Rat Injected with L-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 24. Rechromatography of Combined Fractions 13-30 from the pH 4.0 Column (Figure 23) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F
1 mM, 2 ml ea.
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml)
4 ml each
pH 4.0 TEA-F 0.1 M:0.3M
4 ml each (200ml) (200ml)
CPM X 10^4/Fraction
OH-Kyn
N-A-OH-Kyn
N-A-Kyn
K.A.
X.A.
B6GFL7
Unknown I
(B6GFL8)
B6BFL9
Fraction Number

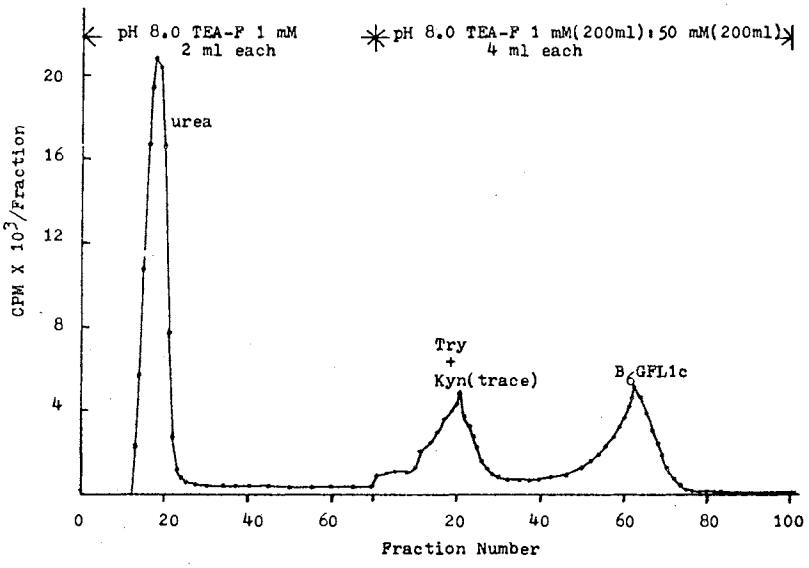

pH 8.0 TEA-F 1 mM
2 ml each
pH 8.0 TEA-F 1 mM(200ml):50 mM(200ml)
4 ml each
CPM X 10^3/Fraction
urea
Try
+
Kyn(trace)
B6GFL1c
Fraction Number

TABLE IV

PROPERTIES OF B_6GFL7* (OR B_6GFD9#) AND B_6GFL9*

	B_6GFL7	B_6GFL9
Rf in BuOH-HAc-H_2O (4:1:5 by vol)	0.15-0.22	0.20-0.22
Rf in 20% (w/v) KCl	0.90-0.94	0.40-0.42
Ehrlich's reagent test	Negative	Negative
Ninhydrin test	Negative	Negative
Fluorescence	Light Blue	Light Blue

*The metabolites were isolated from the urine of B_6-deficient germ-free rat injected with L-tryptophan.

#The metabolite was isolated from the urine of B_6-deficient germ-free rat injected with D-tryptophan.

and normal animals, those that were under B_6-depletion and loaded with L-tryptophan, was observed. However, in this experiment, a significant excretion of X. A., OH-Kyn, K. A., Kyn, N-A-Kyn and N-A-OH-Kyn was observed (Figures 25 and 26). IAA and I-gly were not detected. The X. A. conjugate compound (Unknown I) was also observed (B_6LN7 in Figure 25).

Urinary Metabolites of Indoleacetic Acid-2-^{14}C in B_6-Fed and B_6-Deficient Animals

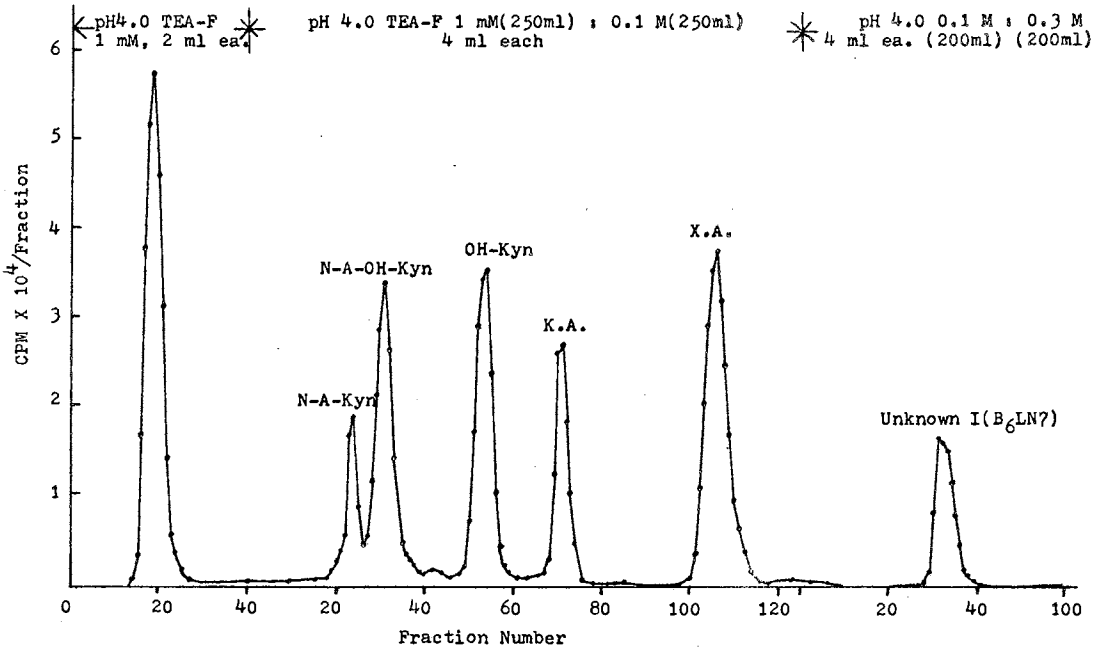
Since I-ald has been reported as an end product from tryptophan through the intermediate, IAA, by the action of oxidase in plants (61, 62, 63, 64), this experiment was intended to determine if IAA is the precursor of I-ald in the mammal. The data suggest that I-ald is not derived from IAA in the rat.

In the normal rat.--Of the injected radioactivity 96% was excreted in the urine, 51% of the total dose remained as the original form, IAA, and 45% was conjugated with glycine as indoleaceturic acid (I-gly) (Figure 27). I-ald was not detected in the urine.

In the B_6-deficient rat.--It was surprising that 85% of the injected IAA-^{14}C was isolated as I-gly, and only 6% of the total dose remained as IAA (Figure 28). Moreover, I-ald was not detected.

Figure 25. Separation of Urinary Tryptophan Metabolites of B_6-Deficient Rat Injected with L-Tryptophan-Methylene-^{14}C on the pH 4.0 DEAE-Cellulose Column.

Figure 26. Rechromatography of Combined Fractions 15-30 from the pH 4.0 Column (Figure 25) on the pH 8.0 DEAE-Cellulose Column.


pH4.0 TEA-F
1 mM, 2 ml ea.
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml)
4 ml each
pH 4.0 0.1 M : 0.3 M
4 ml ea. (200ml) (200ml)
CPM X 10^4/Fraction
N-A-Kyn
N-A-OH-Kyn
OH-Kyn
K.A.
X.A.
Unknown I(B6LN7)
Fraction Number

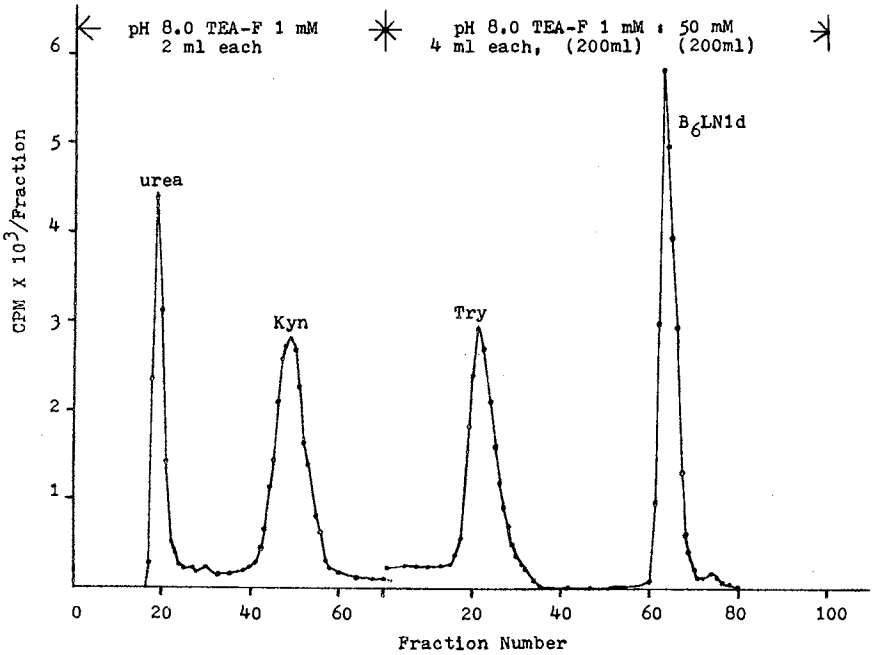

pH 8.0 TEA-F 1 mM
2 ml each
pH 8.0 TEA-F 1 mM : 50 mM
4 ml each, (200ml) (200ml)
CPM X 10^3/Fraction
urea
Kyn
Try
B6LN1d
Fraction Number

Figure 27. Separation of Urinary Indole-3-Acetic Acid Metabolites of Normal Rat Injected with Indole-3-Acetic Acid-2-^{14}C.

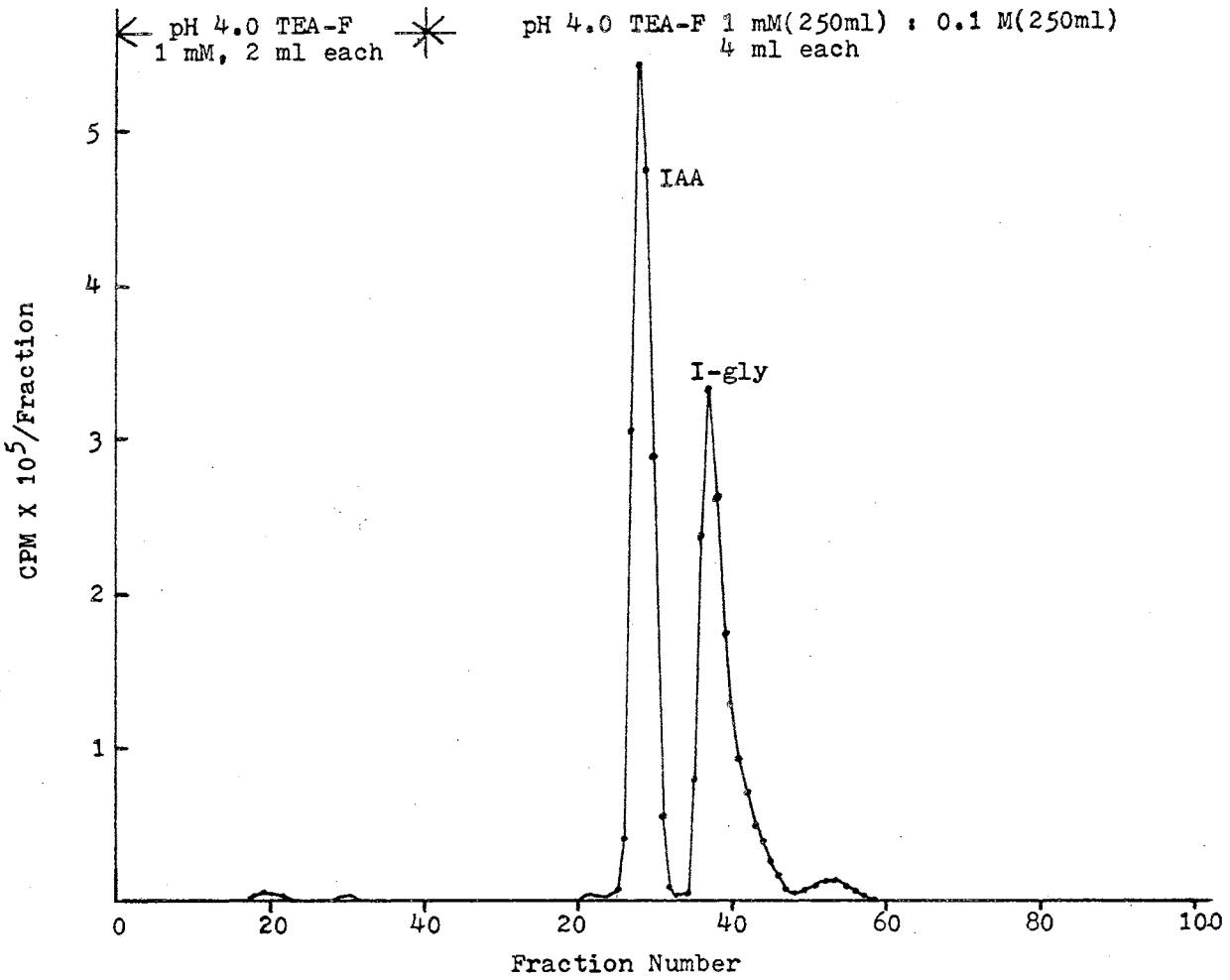
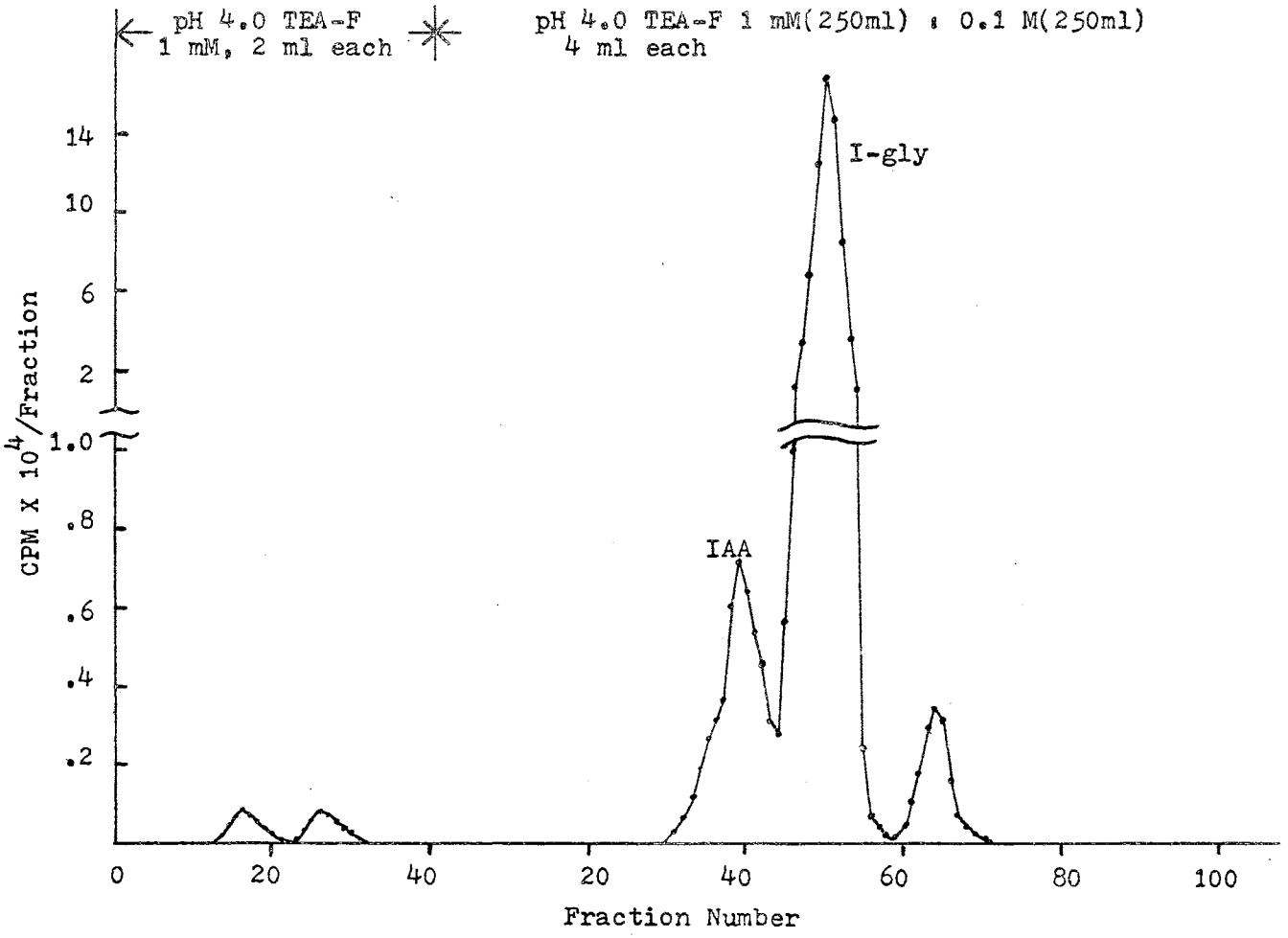

pH 4.0 TEA-F
1 mM, 2 ml each
pH 4.0 TEA-F 1 mM(250ml) : 0.1 M(250ml)
4 ml each
IAA
I-gly
CPM X 10^5/Fraction
Fraction Number
0
20
40
20
40
60
80
100
1
2
3
4
5

Figure 28. Separation of Urinary Indole-3-Acetic Acid Metabolites of B_6-Deficient Rat Injected with Indole-3-Acetic Acid-2-^{14}C.


pH 4.0 TEA-F
1 mM, 2 ml each
pH 4.0 TEA-F 1 mM(250ml) ; 0.1 M(250ml)
4 ml each
I-gly
IAA
CPM X 10^4/Fraction
14
10
6
2
1.0
.8
.6
.4
.2
0
20
40
20
40
60
80
100
Fraction Number

CHAPTER V

DISCUSSION

Very little was known about the mammalian formation from tryptophan of IAA and related indole compounds when this study was initiated. Indeed, only a few reports suggesting the possible pathways for the formation of IAA had appeared. Weissbach et al. (10) reported that the excretion of IAA was increased markedly by L-tryptophan loading. Most of the IAA which was formed by mammalian tissues in vitro arose through transamination involving α-ketoglutarate and pyridoxal phosphate. Mammalian kidney and liver were both found to be capable of decarboxylating L-tryptophan to yield tryptamine. However, the bulk of IAA was presumed to arise from transamination, with smaller amounts arising through decarboxylation.

Since a satisfactory separation technique for urinary indole compounds was not available before this study was initiated, it was first necessary to develop a convenient and reliable method for isolating the radioactive metabolites of tryptophan from the urine. A DEAE-cellulose dual column chromatography system was developed.

The data summarized in Tables I and III demonstrate that D-tryptophan is a more efficient precursor of IAA than is L-tryptophan. In the germ-free and the normal rats after administration of L-tryptophan, the excretion of IAA and I-gly total 0.9% and 0.9% of the total dose, respectively. On the other hand, 12.8% and 6.3% of IAA

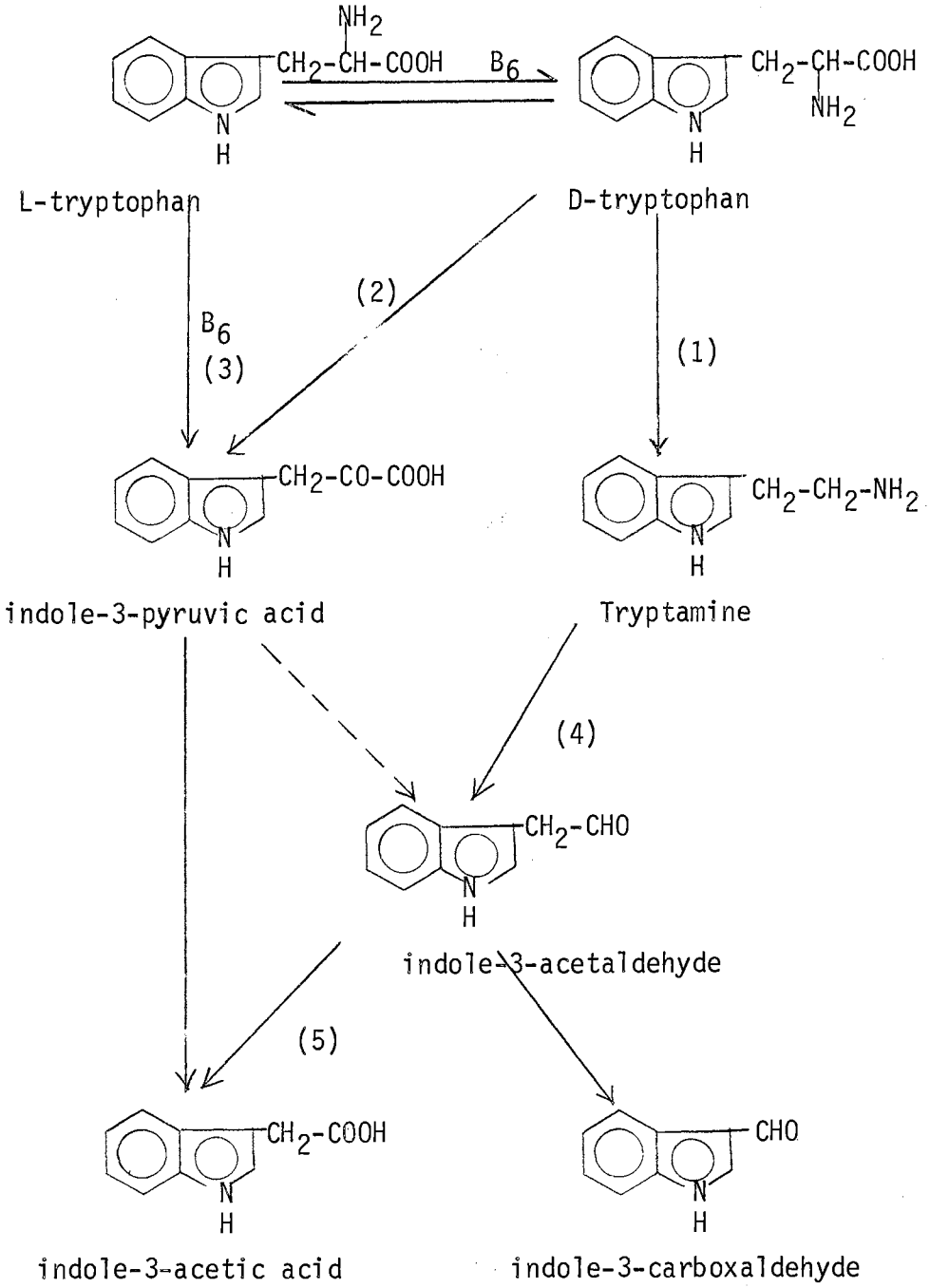
and I-gly are excreted from germ-free and normal rats after administration of D-tryptophan. The transformation of L-tryptophan into IAA was blocked by vitamin B_6-deficiency. However, the result indicated that vitamin B_6 was not required for this transformation from D-tryptophan; 14.3% and 5.8% of the total dosage are excreted from the germ-free and the normal rats after injection of D-tryptophan to vitamin B_6-deficient rats. It is obvious that the mechanism of formation of IAA from D-tryptophan must differ from that of IAA from L-tryptophan in rats.

It is worthy of note that excretion of indole-3-carboxaldehyde either from the vitamin B_6-fed or from the vitamin B_6-deficient animals injected with D-tryptophan was observed. Also, vitamin B_6 seems to have no effect on the formation of indole-3-carboxaldehyde; in germ-free animals 1.9% of the injected dose in the B_6-fed and 6.1% in the B_6-deficient animals were excreted as I-ald; in normal animals, 5.4% in the B_6-fed and 1.7% in the B_6-deficient animals were excreted as I-ald. However, the excretion of this substance was not observed in either vitamin B_6-fed or B_6-deficient animals after the administration of L-tryptophan.

The experimental results indicate that both IAA and I-ald are formed without any effect of pyridoxine from D-tryptophan. Two possible pathways leading to these indole compounds from D-tryptophan can be postulated as shown in Figure 29. In one pathway, D-tryptophan is deaminated by D-amino acid oxidase to indolepyruvic acid, followed by decarboxylation to indoleacetaldehyde which is converted to IAA and I-ald. The decarboxylation step is not significantly decreased in vitamin B_6 deficiency. Another possible path is from D-tryptophan via the intermediate, tryptamine, which is formed without serious

Figure 29. Possible Pathways for the Biosynthesis of Indole-3-Acetic Acid and Indole-3-Carboxaldehyde.

The reactions indicated are: (1) decarboxylation; (2) oxidative deamination; (3) transamination; (4) monoamine oxidase; (5) aldehyde dehydrogenase.


NH2
CH2-CH-COOH
B6
CH2-CH-COOH
NH2
N
H
L-tryptophan
D-tryptophan
(2)
B6
(3)
(1)
CH2-CO-COOH
CH2-CH2-NH2
indole-3-pyruvic acid
Tryptamine
(4)
CH2-CHO
indole-3-acetaldehyde
(5)
CH2-COOH
CHO
indole-3-acetic acid
indole-3-carboxaldehyde

limitation in B_6 deficiency by decarboxylation. The finding of tryptamine in the urine of B_6-deficient animals may support this hypothesis.

It is well known that indolealkylamines which are substrates of monoamine oxidase yield the corresponding indoleacetic acids as their final metabolic products. The whole process (137, 138) comprises two steps: In the first, initiated by monoamine oxidase, the amine undergoes oxidative deamination to the aldehyde, and in the second, catalyzed by aldehyde dehydrogenase, the intermediate aldehyde is oxidized to the acid.

Very recently, Keglevic et al. (139) proposed a pathway for the formation of 5-hydroxyindoleacetic acid and 5-hydroxyindole-3-carboxaldehyde from 5-hydroxytryptamine (serotonin). They reported that the intermediate, 5-hydroxyindoleacetaldehyde, is not stable enough to permit isolation and purification from biological materials. Indeed, the existence of 5-hydroxyindoleacetaldehyde as an intermediate in serotonin metabolism has been unequivocally established (107, 140) using a trapping technique for its detection. Hence, indoleacetaldehyde is most likely an intermediate on the pathway leading to IAA and I-ald.

The interference of vitamin B_6-deficiency in the formation of indole compounds from L-tryptophan is consistent with the report of Weissbach et al. (10). However, the data presented in the thesis do not establish whether the pathway leading to IAA from L-tryptophan proceeds via indolepyruvic acid, or whether L-tryptophan is converted to D-tryptophan prior to further metabolism, or both.

It is worthy of mention that Wightman (70) summarized some possible

pathways involved in the biogenesis of simple indole compounds from DL-tryptophan-methylene-^{14}C in cabbages as shown in Figure 1. He suggested that indole-3-acetonitrile was a central intermediate leading to IAA and I-ald, and that indolepyruvate was not confirmed as an intermediate. His report is partially consistent with the findings of this thesis. Since in Wightman's experiment, DL-tryptophan-methylene-^{14}C was used as the precursor of IAA and the indole compounds, it is possible that the IAA, I-ald and related indole compounds may have been derived chiefly from D-tryptophan, not from L-tryptophan.

The existence of the pyrrolase metabolic pathway for D-tryptophan seems to be indisputable. Indeed, the enzyme which converts D-tryptophan to D-kynurenine has been reported by several workers (28, 29). However, our data show that N-A-Kyn and N-A-OH-Kyn are common excretory metabolites from both D- and L-tryptophan. However, no B_6 effect on their formation was observed in D-tryptophan metabolism, in contrast to an increase of these two metabolites following L-tryptophan administration in vitamin B_6-deficiency.

However, the metabolism of D- and L-tryptophan is significantly different (see Tables I and III). In L-tryptophan metabolism, the pyrrolase metabolic pathway seems to be the major route; on the contrary, the pathway leading to the formation of IAA and the related indole compounds is the major one in D-tryptophan metabolism. Following L-tryptophan administration, a significant increase in the excretory metabolites, such as OH-Kyn, K. A. N-A-Kyn, N-A-OH-Kyn, X. A. and the xanthurenic acid conjugate (Unknown I), is observed in the pyridoxine-deficient animals. These findings are in agreement with previous reports (112, 113, 114).

In 1954, Christensen et al. (117) reported that the uptake of glycine, L-tryptophan and L-methionine into the animal cells was decreased by vitamin B_6-depletion. In the experiments with IAA-2-^{14}C administration, 85% of the total dose was isolated from the vitamin B_6-deficient animal as excretory I-gly. This result could be explained by the findings of Christensen et al. if, in the pyridoxine-deficient condition, the glycine transport into most cells is inhibited, but not that into the cells in which I-gly is formed, thus allowing greater availability of glycine to conjugate with IAA. The ratio of excretory IAA and I-gly from various experimental animals (Table V) can provide the evidence to support this hypothesis.

Finally, the lack of formation of I-ald from IAA may be explained in two ways. First, IAA is not a precursor of I-ald in mammalian metabolism. Second, IAA may not be effectively transported into the cell, in which I-ald is formed and thus I-ald is not observed.

TABLE V

RATIO OF RADIOACTIVITY IN INDOLEACETIC ACID/INDOLEACETURIC ACID IN URINE EXCRETED BY NORMAL AND B_6-DEFICIENT RATS FOLLOWING THE INTRAPERITONEAL INJECTION OF INDOLEACETIC ACID-2-^{14}C, L- AND D-TRYPTOPHAN-METHYLENE-^{14}C

	Germ-free		Normal	
Compound injected	B_6-fed	B_6-def	B_6-fed	B_6-def
Indoleacetic acid			1.1	0.07
D-Tryptophan	1.5	0.6	1.5	0.5
L-Tryptophan	2.0	*	2.0	*

*Indoleacetic acid and indoleaceturic acid were not found.

SUMMARY

A DEAE-cellulose dual column chromatography system was developed to separate tryptophan metabolites. A previously unreported urinary metabolite of tryptophan, indole-3-carboxaldehyde was isolated and identified from the urine of normal, vitamin B_6-deficient, germ-free and vitamin B_6-deficient germ-free rats following intraperitoneal injection of D-tryptophan-methylene-^{14}C. This new metabolite was not detected from the urine of rats injected with either L-tryptophan-methylene-^{14}C or indoleacetic acid-2-^{14}C. The pyridoxine-deficient rats, after administration of L-tryptophan-methylene-^{14}C, excreted abnormally large quantities of 3-hydroxykynurenine, kynurenic acid, N-acetylkynurenine, N-acetyl-3-hydroxykynurenine, xanthurenic acid and a conjugate compound of xanthurenic acid in contrast with the lack of formation of indoleacetic acid and indoleaceturic acid. On the contrary, no significant effect of vitamin B_6-deficiency was observed in the metabolism of D-tryptophan.

The experimental data suggest:

1) Indoleacetic acid and indole-3-carboxaldehyde are mainly derived from D-tryptophan in mammals. The pathways leading to these indole compounds can be postulated as proceeding either via decarboxylation to form tryptamine as the precursor of the central intermediate, indoleacetaldehyde, which leads to indoleacetic acid and indole-3-carboxaldehyde, or by oxidative deamination to indolepyruvic acid followed by decarboxylation to indoleacetaldehyde as the precursor of

indoleacetic acid and indole-3-carboxaldehyde.

2) The pathway leading to indoleacetic acid from L-tryptophan may be either via transamination to form indolepyruvic acid or conversion to D-tryptophan prior to further metabolism.

3) The metabolism of D-tryptophan and L-tryptophan is significantly different. In L-tryptophan metabolism, the pyrrolase metabolic pathway seems to be the major route, on the contrary, the pathway leading to indoleacetic acid and related indole compounds seems to be the major one in D-tryptophan metabolism.

4) The lack of formation of indole-3-carboxaldehyde from indoleacetic acid in rats may be explained in two ways. First indoleacetic acid is not a precursor of indole-3-carboxaldehyde in mammalian metabolism. Second, indoleacetic acid may not be effectively transported into the cell, and thus indole-3-carboxaldehyde formation is not observed.

BIBLIOGRAPHY

1. Herter, C. A., J. Biol. Chem., 4, 238, 253 (1908).

2. Kogl, M., A. Haagen-Smit, and H. Erxleben, Z. Physiol. Chem., Hoppe-Seyler's, 214, 241 (1933).

3. Thimann, K. V., J. Biol. Chem., 109, 279 (1935).

4. Gordon, S. A., and F. S. Nieva, Arch. Biochem. Biophys., 20, 356, 367 (1948).

5. Wildman, S. G., M. G. Ferri, and J. Bonner, Arch. Biochem., 13, 131 (1947).

6. Wildman, S. G., and R. M. Muir, Plant Physiol., 24, 84 (1949).

7. Dannenburg, W. N., and J. L. Liverman, Plant Physiol., 32, 263 (1957).

8. Lantican, B. P., and R. M. Muir, Plant Physiol., 42, 1158 (1967).

9. Gholson, R. K., Thesis, University of Illinois, 1958.

10. Weissbach, H., W. King, A. Sjoerdsma, and S. Udenfriend, J. Biol. Chem., 234, 81 (1959).

11. Kaper, J. M., and H. Veldstra, Biochim. Biophys. Acta, 30, 401 (1958).

12. Leal, J. A., V. G. Lilly, and M. E. Gallegly, Can. J. Microbiol., 14, 595 (1968).

13. Dalgliesh, C. E., Metabolism of the aromatic amino acids., Adv. Protein Chem., 10, 31 (1955).

14. Berg, C. P., J. Biol. Chem., 100, 79 (1933).

15. Woods, D. D., Biochem. J., 29, 640 (1935).

16. Majima, S., Z. Physiol. Chem., 243, 250 (1936).

17. Zenk, M. H., and H. Scherf, Biochim. Biophys, Acta, 71, 737 (1963).

18. Berg, C. P., J. Biol. Chem., 94, 661 (1931-32).

19. du Vigneaud, V., R. R. Sealock, and C. V. Etten, J. Biol. Chem., 98, 565 (1932).

20. Sugahara, M., T. Morimoto, T. Kobayashi, and S. Ariyoshi, Arg. Biol. Chem., 31, 77 (1967).

21. Kotake, Y., and S. Goto, Z. Physiol. Chem., 248, 41 (1937).

22. Behrman, E. J., and A. M. Cullen, Federation Proc., 20, 6 (1961).

23. Martin, J. R., and N. N. Durham, Biochem. Biophys. Res. Commun., 14, 338 (1964).

24. Stanier, R. Y., and M. Tsuchida, J. Bacteriol., 58, 45 (1949).

25. Tashiro, M., K. Tsukada, S. Kobayashi, and O. Hayaishi, Biochem. Biophys. Res. Commun., 6, 155 (1961).

26. Borchers, R., C. P. Berg, and C. E. Whitman, J. Biol. Chem., 145, 657 (1942).

27. Mason, M., and C. P. Berg, J. Biol. Chem., 195, 515 (1952).

28. Loh, H. H., Thesis, State Univ. of Iowa, 1965.

29. Higuchi, K., and O. Hayaishi, Arch. Biochem. Biophys., 120, 397 (1967).

30. Larson, P., Dansk. Botan. Arkiv. 11, 1 (1944).

31. Hemberg, T., Acta Horti, Bergiani, 14, 133 (1948).

32. Gordon, S. A., and F. S. Nieva, Arch. Biochem., 20, 356 (1949).

33. Gordon, S. A., and F. S. Nieva, Arch. Biochem., 20, 367 (1949).

34. Larsen, P., and T. Aasheim, "Plant growth regulation," P. 43 (1961).

35. Henbest, H. B., E. R. H. Jones and G. F. Smith, J. Chem. Soc., 3796 (1953).

36. Larsen, P., Am. J. Botany, 36 32 (1949).

37. Thimann, K. V., Arch. Biochem. Biophys., 44, 242 (1953).

38. Moore, T. C., and C. A. Shaner, Plant Physiol., 42, 1787 (1967).

39. Moore, T. C., and C. A. Shaner, Arch. Biochem. Biophys., 127, 613 (1968).

40. Sequeira, L., and A. Kelman, Phytopathology, 52, 439 (1962).

41. Rudman, D., and A. Meister, J. Biol. Chem., 200, 591 (1953).

42. Hartman, T., and K. Glombitza, Arch. Mikrobiol., 56, 1 (1967).

43. Sukanya, N. K., and C. S. Vaidyanathan, Biochem. J., 92, 594 (1964).

44. Kosuge, T., M. G. Heskett, and E. E. Wilson, J. Biol. Chem., 241, 3738 (1966).

45. Phelps, R. H., and L. Sequeira, Phytopathology, 57, 1182 (1967).

46. O'Neil, S. R., and R. D. DeMoss, Arch. Biochem. Biophys., 127, 361 (1968).

47. Kaper, J. M., and H. Veldstra, Biochim. Biophys. Acta, 30, 401 (1958).

48. Larsen, P., A. Harbo, S. Klungsoyr, and T. Aasheim, Physiol. Plant., 15, 552 (1962).

49. Srivastava, B. I. S., Plant Physiol., 39, 781 (1964).

50. Stowe, B. B., Biochem. J., 61, ix (1955).

51. Magie, A. R., E. E. Wilson, and T. Kosuge, Science, 141, 1281 (1963).

52. Crady, E. E., and F. T. Wolf, Physiol. Plant., 12, 526 (1959).

53. Werle, E., and G. Mennicken, Biochem. Z., 291, 325 (1937).

54. Rodnight, R., Biochem. J., 64, 621 (1956).

55. Robinson, B. J., M. Forman, and F. T. Addicott, Plant Physiol., 43, 1321 (1968).

56. Zenk, V. M. H., Z. Naturforschg., 15b, 436 (1960).

57. Hutzinger, O., and T. Kosuge, Biochem., 7, 601 (1968).

58. Fischer, A., Planta, 43, 288 (1954).

59. Ray, P. M., and G. M. Curry, Nature, 181, 895 (1958).

60. Nogueira, A. P., Arquiv. Inst. Biol. (Sao Paulo), 29, 277 (1962).

61. Tang, Y. U., and J. Bonner, Arch. Biochem., 13, 11 (1947).

62. Wagenknecht, A., and R. Burris, Arch. Biochem., 25, 30 (1950).

63. Racusen, D., Arch. Biochem. Biophys., 58, 508 (1955).

64. Stutz, R. E., Plant Physiol., 33, 207 (1958).

65. Wightman, F., Colloq. Intern, Centre Natl. Rech. Sci. (Paris), No. 123, 191 (1963).

66. Sequeira, L., and T. A. Steeves, Plant Physiol., 29, 11 (1954).

67. Morton, R. A., and N. I. Fahmy, Nature, 182, 939 (1958).

68. Glombitza, K. W., Ber. Deut. Botan. Ges., 78, 83 (1965).

69. Iskrić, S., and S. Kveder, Croat. Chem. Acta, 37, 233 (1965).

70. Wightman, F., Can. J. Botany, 40, 689 (1962).

71. Happold, F. C., and L. Hoyle, Biochem. J., 29, 1918 (1935).

72. Wood, W. A., I. C. Gunsalus and W. W. Umbreit, J. Biol. Chem., 170, 313 (1947).

73. Metzler, D. E., M. Ikawa and E. E. Snell, J. Am. Chem. Soc., 76, 648 (1954).

74. Wada, H., T. Morisue, Y. Sakamoto and K. Ichihara, J. Vitaminology, 3, 183 (1957).

75. Burns, R. O., and R. D. DeMoss, Biochim. Biophys. Acta, 65, 233 (1962).

76. Morino, Y., and E. E. Snell, J. Biol. Chem., 242, 5571 (1967).

77. Sakamoto, Y., M. Uchida and K. Ichihara, Med. J. Osaka Univ., 3, 477 (1953).

78. Izquierdo, J. A., and A. O. M. Stoppani, Brit. J. Pharmacol., 8, 389 (1953).

79. Vickery, H. B., and C. L. A. Schmidt, Chem. Revs., 9, 169 (1931).

80. Udenfriend, S., C. T. Clark and E. Titus, Federation Proc., 12, 282 (1953).

81. Dalgliesh, C. E., and R. W. Dutton, Brit. J. Cancer, 11, 296 (1957).

82. Dalgliesh, C. E., and R. W. Dutton, Biochem. J., 65, 21P (1957).

83. Udenfriend, S., H. Weissbach and A. Sjoerdsma, Science, 123, 669 (1956).

84. Erspamer, V., "Psychotropic Drugs," Elsevier, Amsterdam, 1957.

85. Gal, E. M., and F. D. Marshall, Prog. Brain Res., 8, 56 (1964).

86. Gal, E. M., M. Morgan, S. K. Chatterjee and F. D. Marshall, Biochem. Pharmacol., 13 1639 (1964).

87. Weber, L. J., and A. Horita, Biochem. Pharmacol., 14, 1141 (1965).

88. Gal, E. M., J. C. Armstrong and B. Ginsberg, J. Neurochem., 13, 643 (1966).

89. Grahame-Smith, D. G., Biochem. J., 105, 351 (1967).

90. Hákanson, R., and G. J. Hoffman, Biochem. Pharmacol., 16 1677 (1967).

91. Lovenberg, W., E. Jequier and A. Sjoerdsma, Science, 155, 217 (1967).

92. Peters, D. A. V., P. L. McGeer and E. G. McGeer, J. Neurochem., 15, 1431 (1968).

93. Garattini, S., and L. Valzelli, "Serotonin," Elsevier Co., Amsterdam, P. 29 (1965).

94. Pare, C. M. B., M. Sandler and R. S. Stacey, Lancet, 272, 551 (1957).

95. Pletscher, A., Experientia, 12, 479 (1956).

96. Tabachnick, I. I. A., and A. A. Rubin, Proc. Soc. Exptl. Biol. Med., 101, 435 (1959).

97. Ichihara, K., A. Sakamoto, K. Inamori and Y. Sakamoto, J. Biochem. (Tokyo), 44, 649 (1957).

98. Udenfriend, S., C. R. Greveling, H. Posner, B. G. Refield, J. Daly, and B. Witkop, Arch. Biochem. Biophys., 83, 501 (1959).

99. Clark, C. T., H. Weissbach and S. Udenfriend, J. Biol. Chem., 210, 139 (1954).

100. Buzard, J. A., and P. D. Nytch, J. Biol. Chem., 227, 225 (1957).

101. Giarman, N. J., Federation Proc., 15, 428 (1956).

102. Kameswaran, L., and G. B. West, J. Pharm. Pharmacol., 13, 191 (1961).

103. Huang, I., S. Tannenbaum and D. Y. Y. Hsia, Nature, 186, 717 (1960).

104. Blaschko, H., Biochem. J., 50, 52P (1952).

105. Freyburger, W. A., B. E. Graham, M. M. Rapport, P. H. Seay, W. M. Govier, O. F. Swoap and M. J. Van der Brook, J. Pharmacol., 105, 80 (1952).

106. Udenfriend, S., in F. Brücke (Ed.), Biochemistry of the Central Nervous System, Pergamon, 1959.

107. Weissbach, H., B. G. Redfield and S. Udenfriend, J. Biol. Chem., 229, 953 (1957).

108. Barbato, L., Federation Proc., 20, 237d (1961).

109. Cotzias, G. C., I. Selin and J. J. Greenhough, Science, 120, 144 (1954).

110. Vassella, F., and E. Rossi, Minerva Med., 57, 4124 (1966).

111. Wiss, O., and F. Weber, Med. J. Osaka Univ., 8, 41 (1958).

112. Brown, R. R., M. J. Thornton and J. M. Price, J. Clin. Invest., 40, 617 (1961).

113. Korbitz, B. C., J. M. Price and R. R. Brown, J. Nutr., 80, 55 (1963).

114. Miller, L. T., and H. Linkwiler, J. Nutr., 93, 53 (1967).

115. Buxton, J., and H. M. Sinclair, Biochem. J., 62, 27P (1956).

116. Weissbach, H., D. F. Bogdanski, B. G. Redfield and S. Udenfriend, J. Biol. Chem., 227, 617 (1957).

117. Christensen, H. N., T. R. Riggs and B. A. Coyne, J. Biol. Chem., 209, 413 (1954).

118. Wieland, T., and G. Hőrlein, Ann., 591, 192 (1955).

119. Dalgliesh, C. E., Biochem. J., 64, 481 (1956).

120. Armstrong, M. D., K. N. F. Shaw, M. J. Cortatowski and H. Singer, J. Biol. Chem., 232, 17 (1958).

121. Powell, L. E. Nature, 200, 79 (1963).

122. Benassi, C. A., F. M. Veronese and A. De antoni, Clin. Chim. Acta, 17, 383 (1967).

123. Schlossberger, H. G., H. Kuch and I. Buhrow, Z. Physiol. Chem., 333, 152 (1963).

124. Gordon, S. A., and R. P. Weber, Plant Physiol., 26, 192 (1951).

125. Ehrlich, F., and K. A. Jacobsen, Ber. deut. Chem. Ges., 44, 888 (1911).

126. Chen, N. C., and R. K. Gholson, Anal. Biochem., (in preparation).

127. Ratliff, C. R., Am. J. Med. Technol., 28, 83 (1962).

128. Coppini, D., C. A. Benassi and M. Montorsi, Clin. Chem., 5, 391 (1959).

129. Waller, G. R., and L. M. Henderson, J. Biol. Chem., 236, 1186 (1961).

130. Shaw, J. T., and F. T. Ryson, J. Am. Chem. Soc., 78, 2538 (1956).

131. Shriner, R. L., R. C. Fuson and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 5th ed., p. 253, John Wiley and Sons, Inc., New York, 1964.

132. Waller, G. R., Proc. Okla. Acad. Sci., 47, 271 (1968).

133. Li, H. Y., J. Walden, D. Etter and G. R. Waller, Proc. Okla. Acad. Sci. 49, 1968 (In Press).

134. Baglioni, C., P. Fasella, C. Turano and N. Siliprandi, Biochem. J., 74, 521 (1960).

135. Price, J. M., and L. W. Dodge, J. Biol. Chem., 223, 699 (1956).

136. Roy, J. K., and J. M. Price, J. Biol. Chem., 234, 2759 (1959).

137. Blaschko, H., Pharmacol. Rev., 4, 415 (1952).

138. Erspamer, V., J. Physiol., 127, 118 (1955).

139. Keglevic, D., S. Kveder and S. Iskrić, Adv. Pharmacol., 6, 79 (1968).

140. Udenfriend, S., E. Titus, H. Weissbach and R. E. Peterson, J. Biol. Chem., 219, 335 (1956).

VITA

Norman Chea-san Chen

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE CONVERSION OF D- AND L-TRYPTOPHAN TO INDOLE COMPOUNDS IN THE RAT

Major Field: Chemistry

Biographical:

Personal Data: Born in Kausiung City, Taiwan, October 26, 1935, the son of San-shi and San-wu Chen.

Education: Graduated from Taiwan Provincial Kausiung High School, Kausiung City, Taiwan, in 1953; received the Bachelor of Science degree from the Taiwan Provincial Chung-Hsing University, Taichung, Taiwan, in Agricultural Chemistry in June, 1958; received the Master of Agriculture degree from the University of Tokyo, Tokyo, Japan, in Agricultural Chemistry in October, 1964; completed the requirements for the Doctor of Philosophy degree in August, 1969.

Professional Experience: Served as an assistant agricultural chemist in Taiwan Sugar Experiment Station, Tainan, Taiwan, March, 1960, to March, 1962; served as a graduate research assistant, Biochemistry Department, Oklahoma State University, 1965-1969.

Professional Organizations: The American Chemical Society, Society of the Sigma Xi.